2012

Design, Synthesis, and Anticancer Activity of Ruthenium Complexes

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Dr. John Anthony, Director of Graduate Studies
DESIGN, SYNTHESIS, AND ANTICANCER ACTIVITY OF RUTHENIUM COMPLEXES

Thesis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Arts and Sciences at the University of Kentucky

By
Brock S. Howerton

Committee Chair: Dr. Edith Glazer, Professor of Chemistry,
Committee Members: Dr. David Atwood, Professor of Chemistry,
Dr. Rolf Craven, Professor of Molecular and Biomedical Pharmacology

Lexington, Kentucky
2012

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DESIGN, SYNTHESIS, AND ANTICANCER ACTIVITY OF RUTHENIUM COMPLEXES

Ruthenium complexes show promise as light activated photodynamic therapy (PDT) prodrugs. Strained octahedral complexes were synthesized that produce a cytotoxic species upon light activation. pUC19 DNA damage in vitro experiments were carried out to determine the type of damage observed. In vivo cell experiments were carried out on the non-small lung cancer A549 cell line to determine the phototherapeutic window of the synthesized complexes. One mechanism of drug resistance via elevated levels of glutathione was addressed through in vitro binding studies carried out with UV-Vis spectroscopy and in vivo glutathione titrations in the A549 cell line. Several complexes were shown to be potential PDT agents with light-activated activities greater than cisplatin and 10-100 fold lower dark toxicities.

KEYWORDS: ruthenium, cancer, light activated, DNA damage, cytotoxicity
DESIGN, SYNTHESIS, AND ANTICANCER ACTIVITY OF RUTHENIUM COMPLEXES

By

Brock S. Howerton

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Date
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### Abbreviations

**A**  | adenosine  

**A549**  | adenocarcinomic human alveolar basal epithelial cells  

**aq.**  | aqueous  

**bpy**  | 2,2'-bipyridine  

**bc**  | 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline  

**bcds**  | 2,9-dimethyl-4,7-diphenyl-sulfonate-1,10-phenanthroline  

**biq**  | 2,2'-biquinoline  

**2,2'-biq-3,3'-dca**  | 3,3'-dicarboxylic acid-2,2'-biquinoline  

**bp**  | 4,7-diphenyl-1,10-phenanthroline  

**bpds**  | 4,7-diphenyl-sulfonate-1,10-phenanthroline  

**C**  | cytidine  

**ct**  | calf thymus  

**cyclobpy**  | 2-phenyl pyridine  

**cyclophen**  | 7,8-benzoquinoline  

**cont.**  | continued  

**d**  | doublet
dd  double of doublets

dmbpy  6,6'-dimethyl-2,2'-bipyridine

DMEM  Dulbecco's modified Eagle's Medium

dmdppz  dimethyl- dipyrido[3,2-2',3'-c]phenazine

dmdpq  dimethyl- dipyrido[3,2-f:2',3'-h-quinoxaline]

dmphenn  2,9-dimethyl-1,10-phenanthroline

DNA  deoxyribonucleic acid

dppz  dipyrido[3,2-2',3'-c]phenazine

dpq  dipyrido[3,2-f:2',3'-h-quinoxaline]

ESI-MS  electrospray ionization mass spectrometry

G  guanosine

GSH  glutathione

HPLC  high pressure liquid chromatography

HL60  human promyeloxytic leukemia cell line

8HQ  8-hydroxyquinoline

IC50  Concentration of a compound that induces 50% growth

K562  myelogenous leukemia cell line
m multiplelet
MC metal centered
mmol millimole
2Me8HQ 2-methyl-8-hydroxyquinoline
MLCT metal-to-ligand-charge-transfer
m/z mass to charge ratio
ML2 myeloblastic leukemia cell line
NMR nuclear magnetic resonance
PBS phosphate buffered saline
PDT photodynamic therapy
phen 1,10-phenanthroline
Pt platinum
Ru ruthenium
RNA ribonucleic acid
s singlet
t triplet
T thymidine
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>U937</td>
<td>histiocytic lymphoma cell line</td>
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</table>
Chapter 1: Introduction

In 2008, 12.7 million new patients were diagnosed with cancer and 7.6 million patients died from cancer related issues. The disease does not discriminate between societies, afflicting economically developed and developing countries alike. The term cancer applies to a broad swath of diseases characterized by the inability of an abnormal cell to regulate growth, allowing it to potentially form tumors. Disruption of the regulation of normal tumor suppression genes and activation of oncogenes lead to this abnormal growth and fast track the development of the disease. The combination of over expression of cell growth promoters and increased cellular survival mechanisms allow the abnormal cells to proliferate.

Common cancer therapies include surgery, radiotherapy, and chemotherapy. Combinations of the three are common based on the location of the cancer, risk factors, and potential for successful outcomes. Chemotherapy involves the use of antineoplastic drugs to kill cells that divide rapidly. Current drugs in the clinic can be divided into categories based on their mechanism of action to prevent replication of rogue cells, and many cause DNA damage leading to apoptosis. Drug categories include alkylating agents, antimetabolites, anthracyclines, plant alkaloids, and topoisomerase inhibitors.

Approximately 50% of people undergoing chemotherapy are treated with the alkylating agent, cisplatin. Cisplatin is used to treat a variety of cancers including testicular, ovarian, head, and neck cancers. The drug works by covalently binding
to DNA, rendering the cell unable to undergo cellular division. When DNA repair mechanisms fail to work, apoptosis results.\textsuperscript{12} Cisplatin and similar platinum complexes favor the N7 position of pyrimidines and the N3 position of purines (Figure 1.1).\textsuperscript{13}

Figure 1.1: Base pairs shown numbered and hydrogen bonded

<table>
<thead>
<tr>
<th>Guanine (G) – Cytosine (C)</th>
<th>![Diagram of Guanine (G) – Cytosine (C)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine (A) – Thymine (T)</td>
<td>![Diagram of Adenine (A) – Thymine (T)]</td>
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</table>

Cisplatin is capable of platinating double stranded DNA through intrastrand and interstrand mechanisms.\textsuperscript{14} The majority of cytotoxic interactions arise from the intrastrand cross-linking of adjacent purines. Guanosine is preferred over adenosine, and cisplatin favors intrastrand binding of adjacent guanosines, but will also coordinate adenosines adjacent to guanosine. Interstrand complexes are lesser realized and provide similar damage.\textsuperscript{15} All of these structures distort the DNA structure and activate cellular DNA damage mechanisms such as the expression of p53, and apoptosis.\textsuperscript{16}
Drug resistance for platinum compound chemotherapy is thought to arise from many mechanisms, including detoxification by cellular sulfur-containing compounds. Glutathione is a natural tripeptide found in the cytosol of almost all cells in concentrations up to 16 mM. The sulfur rich compound is a good ligand for platinum according to Pearson’s hard soft acid base theory. Other sulfur containing metal detoxification agents such as metallothionein have been found in similar concentrations in cisplatin-insensitive cells. These detoxifying agents interfere with the chemotherapeutic role of platinum compounds and are a substantial contributor to increased drug resistance. Several derivatives of cisplatin such as oxaliplatin and carboplatin have been synthesized with the goal to thwart resistance, decrease toxicity and improve activity. Figure 1.2 shows several structures of platinum compounds that are currently in clinical trials and commercially available for therapeutic use. These platinum derivatives work through the same DNA damage mechanism and share dose limiting toxicities and adverse side effects observed with cisplatin. These side effects include nephrotoxicity, nausea, ototoxicity, and electrolyte disturbance.
Figure 1.2: Current platinum chemotherapeutic agents

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Complex Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>carboplatin</td>
<td><img src="image" alt="carboplatin Image" /></td>
</tr>
<tr>
<td>oxaliplatin</td>
<td><img src="image" alt="oxaliplatin Image" /></td>
</tr>
<tr>
<td>AMD473</td>
<td><img src="image" alt="AMD473 Image" /></td>
</tr>
<tr>
<td>JM216 (satraplatin)</td>
<td><img src="image" alt="JM216 Image" /></td>
</tr>
</tbody>
</table>

Despite the toxicity and side effects of cisplatin and other platinum derivatives, these drugs (in combination with other therapeutics) are quite successful in treating a variety of cancers.\textsuperscript{28,29,30} Building on this success, other metal centered drugs have been synthesized in an attempt to minimize adverse effects.\textsuperscript{31,32} Several metals including ruthenium, rhenium, and osmium have been investigated as possible chemotherapeutics.\textsuperscript{33} Ruthenium compounds are a potential alternative to their platinum counterparts due to the decreased toxicity and variable oxidation states accessible under physiological conditions.\textsuperscript{34} Two ruthenium drugs, NAMI-A and KP1019 are currently in clinical trials (Figure 1.3).\textsuperscript{35,36}
Figure 1.3: Ruthenium drugs currently in clinical trials

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Complex Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAMI-A</td>
<td><img src="image" alt="NAMI-A Structure" /></td>
</tr>
<tr>
<td>KP1019</td>
<td><img src="image" alt="KP1019 Structure" /></td>
</tr>
</tbody>
</table>

NAMI-A is an arene Ru(III) drug that is used for treatment of non-small cell lung cancer through reducing metastases weight without affecting the primary tumor. It is well tolerated by patients in clinical studies.\(^{37}\) KP1019 is activated by reduction from Ru\(^{III}\) to Ru\(^{II}\) in hypoxic tumor tissues by reducing sulfur rich biomolecules such as glutathione.\(^{34}\) KP1019 has been used in clinical trials for colorectal cancer and is also well tolerated in patients.\(^{36}\)

Ruthenium polypyridyl complexes have also been investigated for their potential chemotherapeutic qualities. These complexes are well studied, and derivatives of the ruthenium polypyridyl complex have been synthesized to target DNA.\(^{34,38}\) Due to the rich synthetic nature of ruthenium, several complexes containing ligands with high DNA affinity have been synthesized. The groove binding and
metallo-intercalating ability of 1,10 phenanthroline (phen) and dipyrido [3,2-f: 2', 3'-h-quinoxaline] (dpq) (see Table 2.1) are well known.\textsuperscript{39}

NAMI-A and KP1019 provide novel chemotherapeutics outside of cisplatin and display how transition metals can provide several interesting complex structures capable of leading to apoptosis. Alternatively, the DNA binding ruthenium compounds in the literature provides an alternative to platinum drugs with a non-covalent mechanism of DNA interactions. The problem with all these approaches, however, is the lack of specificity, as these drugs do not discriminate between healthy and cancerous cells.

To improve selectivity and decrease toxicity, photoactive complexes can increase the therapeutic window of antitumor drugs. For this reason photodynamic therapy (PDT) can be used to treat localized tumors with laser-based fiber-optic devices.\textsuperscript{40} PDT is used to treat a variety of cancers accessible with a light source including lung, superficial gastric, cervical, bladder, head, and neck. Patients utilizing PDT benefit from the relatively non-invasiveness of the treatment that can often be administered in an outpatient setting.\textsuperscript{41} This targeted treatment reduces the side effects associated with traditional chemotherapy and because dose-limiting toxicity is nonexistent, toxicity is only induced in the targeted tissues when light activated. Repeated treatments are therefore possible. Photofrin is clinically used to treat early and advanced stage lung cancer and Foscan is used in treatment of palliative head and neck cancer (see Figure 1.4).\textsuperscript{42,43}
Figure 1.4: Photosensitizers available for chemotherapeutic use

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Complex Structure</th>
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<tbody>
<tr>
<td>Photofrin</td>
<td><img src="image" alt="Photofrin" /></td>
</tr>
<tr>
<td>Foscan</td>
<td><img src="image" alt="Foscan" /></td>
</tr>
<tr>
<td>Levulan</td>
<td><img src="image" alt="Levulan" /></td>
</tr>
<tr>
<td>Metvix</td>
<td><img src="image" alt="Metvix" /></td>
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Topical photosensitizers such as Levulan and Metvix are biosynthetic precursors to photofrin, and are used in the clinic to treat actinic keratosis. The benefit of these drugs lies in the mechanism of action. These catalytic compounds form singlet oxygen as they absorb the light energy, becoming excited and transferring the energy to triplet excited oxygen ($^3$O$_2$). This energy transfer causes one of the unpaired valence electrons to flip, fueling the production of the cytotoxic
singlet oxygen ($^{1}\text{O}_2$). Added selectivity arises from the inability of the highly reactive, short-lived species to diffuse across more than one membrane, causing minimal damage to normal tissue. The drawback to the singlet oxygen mechanism arises from the apoxic nature of malignant and more aggressive cancer cells.

Photoactivatable metal complexes have been investigated as alternative PDT therapeutics to porphyrin based photosensitizers. Photoactive octahedral Pt$^{IV}$ complexes have been investigated due to their ability when light activated to be reduced to their cytotoxic Pt$^{II}$ species. These complexes show better aqueous solubility and increased therapeutic indexes with reduced toxicity. One drawback, however, is the need to activate the Pt$^{IV}$ species with high energy UV light. Polypyridyl ruthenium complexes have also been investigated due to the fact that various structures can be readily synthesized and possess tunable absorption properties. Long wavelength light is desired in PDT due to its increased tissue penetration. Low energy transitions that absorb at longer wavelengths in the MLCT (metal-to-ligand-charge transfer) region are an important attribute in the design of the metal-based compounds. Various groups have developed several metal complexes that display light induced cytotoxicity through oxygen independent mechanisms.

Due to the rich nature of the synthetic chemistry associated with ruthenium compounds, and the promising clinical results of NAMI-1, KP1019 and photoactivatable platinum complexes, alternative light-active ruthenium complexes hold promise as potential anti-cancer agents. The goal of this research project is to
produce novel ruthenium compounds that display low to no activity in the dark in both *in vivo* and *in vitro* experiments, but can be turned on by light to become potent cytotoxic agents. We aim to make efficient light activated complexes that display high DNA binding, crosslinking, or other forms of DNA damage, resulting in toxicity in cancer cells. The primary factor considered for light-activated agents is the ratio of the activity in light vs. in the dark, which is termed the phototherapeutic ratio. This is a direct measurement of the therapeutic window for PDT. Several compounds have been synthesized, characterized, and screened in cancer cells for beneficial anti-tumor behavior in this work. The goals defined at the onset of the project included light-activated potencies comparable to cisplatin, and a 10-fold phototherapeutic ratio, allowing for the potential of significant reduction in side-effects in future clinical applications. The progress towards achieving these goals is described in the following chapters. The thesis is divided into the following chapters: Design, synthesis, *in vitro* and *in vivo* characterization of the complexes synthesized for this thesis. Several scientists contributed to this work and are appropriately cited for the work they contributed.
Chapter 2: Design and Synthesis

1: Design

The photochemistry of ruthenium polypyridyl complexes such as [Ru(bpy)$_3$]$^{2+}$ have been extensively studied. The unique combination of chemical stability, redox properties, excited-state reactivity, luminescence emission, and excited-state lifetime of [Ru(bpy)$_3$]$^{2+}$ and its derivatives has garnered the attention of researchers across multiple disciplines. Ru(II) polypyridyl complexes play key roles in multiple research areas such as photophysics, photocatalysis, and electron and energy transfer. The photochemical and photophysical nature of these compounds is of interest to our group and will be the main focus of this chapter. The hypothesis of this project is that light-activated Ru(II) complexes can be designed to eject ligands upon irradiation, producing ligand deficient systems that will react with and crosslink DNA, like cisplatin. The application of these systems is for the development of photo-responsive chemotherapeutic agents. The key feature that controls this photochemical reaction is intramolecular strain, generating distorted Ru(II) complexes that undergo photochemical reactions with low energy, visible light.

Unstrained Ru(II) polypyridine complexes such as [Ru(bpy)$_3$]$^{2+}$ contain ligands with $\sigma$ donor orbitals that are localized on the nitrogen atoms and $\pi$ donor and $\pi^*$ acceptor orbitals delocalized on the aromatic rings. Ru(II) polypyridyl complexes are typically orange to red, due to their absorption of visible light. The
absorption spectrum of [Ru(bpy)$_3$]$^{2+}$ is shown in Figure 2.1 with the electronic transitions assigned.

Figure 2.1: Electronic absorption spectrum of a) [Ru(bpy)$_3$]$^{2+}$ and b) GL002 [Ru(bpy)$_2$-6,6’-dmbpy]$_2^{2+}$ in dH$_2$O.

The intense band at 285 nm results from spin-allowed, ligand centered (LC) $\pi$ to $\pi^*$ transitions. The intense metal-to-ligand charge transfer (MLCT) bands at 240 and 450 nm result from spin allowed $d$ to $\pi^*$ transitions. The less intense shoulder bands at 320 and 340 nm result from metal-centered (MC), $\pi_M$ to $\sigma_M$ transitions.

Figure 2.2a shows a simplified Jablonski diagram for excitation of an electron by following absorption of a photon with promotion from the ground state to the $^1$MLCT.
Figure 2.2: Electronic transitions of (a) unstrained d$^6$ Ru(II) complexes and (b) strained d$^6$ Ru(II) complexes.

(a)

(b)

Once the $^1$MLCT excited state is populated, intersystem crossing efficiently funnels electrons to the lower $^3$MLCT excited state. Radiative relaxation from the $^3$MLCT to the ground state of [Ru(bpy)$_3$]$^{2+}$ produces an intense emission band at 610nm and is shown in Figure 2.3a.
Figure 2.3: Emission profile of a) [Ru(bpy)₃]²⁺ and b) [Ru(bpy)₂dmbpy]²⁺.

Long luminescent lifetimes of 800 ns are reported for the transition from the ³MLCT excited state to ground state with a quantum efficiency of ~0.06. Population of the ³MC state for [Ru(bpy)₃]²⁺ is inefficient, producing a poor quantum yield for photodecomposition on the order of 10⁻⁵-10⁻². For unstrained d⁶ Ru(II) complexes such as [Ru(bpy)₃]²⁺, population of the ³MC state is inefficient due to the large energy gap between the ³MLCT and ³MC, resulting mainly in emission from the ³MLCT state.

Absorption profiles similar to [Ru(bpy)₃]²⁺ are observed in strained d⁶ Ru(II) polypyridine complexes. The emission profile of the complex Ru(bpy)₂-6,6'-dmbpy, GL002, is shown in Figure 2.3b. In contrast to standard octahedral Ru(II) complexes, strained complexes undergo fast radiationless deactivation to the ground state. This occurs through ligand dissociation reactions when the ³MC state is lowered in energy so that it can be accessed thermally from the ³MLCT state. Synthesis of sterically strained ruthenium complexes decreases the ³MLCT to ³MC energy gap, allowing population of the ³MC excited state, resulting in cleavage of the Ru-N bond. Figure 2.2b shows this in an altered Jablonski diagram, and depicts how electrons
are successfully shuttled to the $^3$MC excited state orbitals. The efficient population of the $^3$MC state is manifest in the decreased intensity of the emission spectra shown in Figure 2.3b.

Upon photo-excitation of the complex, a bond is broken to a bipyrene ligand, and a monodentate bipyridine intermediate with a ruthenium that is pentacoordinated forms. In the presence of excess electron donating ligands such as chloride ions or solvent molecules, new bonds to the ruthenium can form. At this point, are-coordination process can occur, reforming the Ru-N bond or alternatively, the single remaining bond to the bipyridine ligand can be broken, forming a Ru(II) complex with two chloride or two solvent ligands. The re-coordination process is possible with unstrained octahedral complexes such as [Ru(bpy)$_3$]$^{2+}$, while ligand loss prevails in the model compound GL002, due to the steric clash of the methyl groups.

Photolabile model complexes with the Ru(bpy)$_2$ backbone containing sterically crowding ligands such as 6,6′-dimethyl-2,2′-bipyridine have been prepared to promote the population of the $^3$MC excited state, leading to the complete dissociation of the ligand. This generates the ligand deficient, bis-bipyridine Ru(II) complex capable of cross-linking DNA. Several photo-active complexes utilizing the Ru(bpy)$_2$ backbone have been synthesized including GL002, 003, 006, 007, 008, 010, and 014 (see Table 2.2). The rate at which the complexes photo-dissociate can be tuned through the choice of ligands. The complex GL002 has fast photoejection kinetics ($t_{1/2}=1.9$ minutes, see Table 3.1) due to the free rotation about the 2,2′-
carbon-carbon bond of 6,6’-dimethyl-2,2’-bipyridine. In contrast, addition of rigid ligands to the Ru(bpy)$_2$ backbone slows the ejection process. For example, when the 2,9'-dimethyl-1,10-phenanthroline ligand is incorporated (see GL007, Figure 2.5), $t_{1/2}$ of 112.8 minutes is obtained, as shown in Table 3.1. This is attributed to the ligands’ ability to re-coordinate the metal, due to its rigid chelating structure.

The DNA affinity of the complexes can also be tuned through the addition of intercalating ligands such as dpq (dipyrido[3,2-f:2’,3’-h]quinoxaline) and dppz (dipyrido[3,2-2’,3’-c]phenazine) (see Table 2.1). These planar ligands are known to intercalate into the base stack of DNA.$^3$ Complexes GL003, 009, 010, 021 and 039 were synthesized to test if photoejecting complexes with DNA intercalating ligands are more potent than compounds with lower DNA affinity. Unstrained complexes GL009 and 021 contain dpq and dppz ligands that display binding to pUC19 DNA

in vitro, as discussed in Chapter 4. The photoejectable analogues of these complexes GL003, 010, and 039 were synthesized to explore their biological activity in vitro and in vivo.

Complexes with different overall charge states and alternative backbones have been prepared to determine how these factors change the in vitro and in vivo experimental results. Ruthenium compounds with different overall charges such as GL005 (overall charge of -2) and 008 (overall charge of 0) display different cytotoxic properties. Decreased affinity to the negatively charged DNA backbone is thought to account for some of the differences in biological activities. Complexes with different
Ru(dmphen)$_2$ backbones have also synthesized. GL018, 019, 022, 023, and 039 are examples of these complexes (see Table 2.2).

Complexes GL018 and 019 contain hydroxyquinoline ligands that are known to be cytotoxic. These complexes were designed to test the hypothesis that complexes could be generated that would create two active species – the ligand deficient Ru(II) complex, and the liberated ligand. Studies have shown that hydroxyquinoline and its derivatives have anti-proliferative and cytotoxic effects in leukemia cells lines. They inhibit RNA synthesis in *E. coli* bacteria and iron complexes containing these ligands have been shown to be effective in the U937, K562, ML2, and HL60 cell lines. In addition, hydroxyquinoline derivatives chelate copper, and acts as angiogenesis and proteasome inhibitors in prostate cancer cell lines.

An effort has been made to produce ruthenium complexes that absorb at longer wavelengths, as longer wavelength (lower energy) light can penetrate deeper into tissues. This would provide access to tumors previously unreachable via photodynamic therapy. Addition of the biquinoline (biq) ligand to the Ru(phen)$_2$ backbone (see Table 2.2) produces a 60 nm red shift in the UV/Vis profile of complex GL011 (see Figure 3.2). Alternatively, cyclometallating ligands 2-phenylpyridine and 7,8-benzoquinoline coupled with the Ru(bpy)$_2$ backbone produced complexes GL034 and 035 (see Table 2.2). These cyclometallated systems display 100 nm red shifts in their corresponding UV-Vis profiles (Figure 3.1). Unfortunately these compounds do not eject as they are unstrained, and are unable to populate the $^3$MC excited state. In the future, strained and photo-active analogues
of these systems should be synthesized to determine their *in vitro* and *in vivo* properties. Finally, oxygen containing hydroxyquinoline ligands also act to lower the energy of the MLCT excited state, potentially red-shifting the absorption of the complexes further into the PDT therapeutic window. Derivatives of complexes containing the backbones presented in this work with different quinolines should be synthesized to evaluate their phototherapeutic ratio.

2. Synthesis

Several scientists contributed to the synthesis chapter, including Dr. Edith Glazer, Erin Wachter, and Emily Hall.

Several ruthenium complexes were synthesized to gain an understanding of how the structural characteristics affect the photophysics and photochemistry of the complexes and their *in vitro* activities with pUC19 DNA (Chapter 4) and *in vivo* potencies in the A549 cell line (Chapter 5). Complexes that produce an activated species upon exposure to light were prepared to observe differences in light and dark activity with DNA.

The general preparation of these complexes begins with the synthesis of the ligands used (Figure 2.4) and then generating the Ru(L)2Cl2 starting material (see Figure 2.5). The synthetic nature of ruthenium (see Chapter 1) allows efficient combinatorial design. Mono ligands ejected from synthesized complexes upon light activation that show toxicity can be incorporated into the *bis*-Ru backbone resulting in massive libraries that can be screened against the cell line. Several ligands
utilized in this thesis were synthesized to test how structural planarity of the ligand effect the photoejection kinetics, \textit{in vitro}, and \textit{in vivo} results. The general synthetic pathway to produce the ligand(s) is shown in Figure 2.4. These are good reactions, with yields of 60% or greater.\textsuperscript{8,9}

Figure 2.4: General synthesis of extended polyaromatic ligands

Once the appropriate ligands are synthesized, the desired ruthenium complex starting material can be produced with the reaction scheme shown in Figure 2.5 in good yields.\textsuperscript{10}

Figure 2.5: Synthesis of the ruthenium starting material with two bidentate ligands

With the synthesized starting materials, modular, high yielding complexes can be produced.\textsuperscript{11} The reactions and subsequent purification are carried out in diminished light conditions to prevent photo-degradation of the complexes.
Figure 2.6: Addition of a third ligand to produce efficient phototherapeutic agents

![Diagram showing the addition of a third ligand to produce efficient phototherapeutic agents.]

General preparation of complexes with Ru(L)$_2$Cl$_2$ backbone:

A solution of RuCl$_3$ (3.8 mmol, 1 g), free ligand L (7.6 mmol, variable g), LiCl (57 mmol, 2.4 g) and ascorbic acid (4.2 mmol, 0.74 g) were added to 25 mL dry DMF and refluxed at 150 °C for 12 hours. The solution was allowed to cool to room temperature, and the purple product was precipitated with a mixture of cold acetone/ether (50 mL each). The precipitate was collected by vacuum filtration and washed with acetone/ether and dried under vacuum. The yield of Ru(phen)$_2$Cl$_2$ is 65% and Ru(dmphen)$_2$Cl$_2$ is 71%.

General preparation of complexes with Ru(L)$_2$L’-2PF$_6$:

A solution of 0.38 mmol Ru(L)$_2$Cl$_2$ (0.1 g) and 0.42 mmol L’ (variable g) was added to 4 mL ethylene glycol in a pressure tube and heated at 150 °C for ~4 hours. The solution was allowed to cool and poured into 50 mL of dH$_2$O. A saturated solution of aq. KPF$_6$ was added to precipitate the complex as the PF$_6$ salt. The precipitate was collected by vacuum filtration and washed with water (50 mL) and...
ether (50 mL) and dried under vacuum. A non-emissive, dim spot on thin layer chromatography (TLC) (0.1% saturated KNO$_3$/20%H$_2$O/80%MeCN on silica plates) is evidence that the strained complex has formed (see Figure 2.2b).

For unstrained complexes, the starting materials were added to 50:50 water: ethanol and refluxed at 100 °C for 4 hours. The solution was poured into ~50 mL dH$_2$O and the excess ligand was extracted with methylene chloride. Saturated aq. KPF$_6$ was added and the PF$_6$ salt was extracted in methylene chloride. The layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure. A bright, emissive spot observed using TLC conditions described above is evidence that the unstrained complex has formed (see Figure 2.2a). Similar purification of the complexes was carried out using flash chromatography. Complexes were loaded onto the column in acetonitrile and eluted with a ramping gradient of 0.1% saturated KNO$_3$/20%H$_2$O/80%MeCN. The synthesized complexes typically elute at 9% dH$_2$O. Solvent was removed under reduced pressure and reconstituted in 25 mL of dH$_2$O. A saturated aq. solution of KPF$_6$ was added, and the metal complex was extracted into methylene chloride. Removal of the solvent under reduced pressure gave pure PF$_6$ complex salts.

General procedure to counter-ion exchange to produce water-soluble complex salts:

Saturated tetra-n-butyl ammonium chloride (1 g in 5 mL dry acetone) was added to the PF$_6$ complex salts dissolved in minimal acetone. The resulting precipitate was filtered in a long stem hersch funnel packed with glass wool. The
precipitate was washed with acetone and eluted with acetonitrile. The solvent was removed under reduced pressure to yield the water soluble Ru(L)$_2$L’-2Cl salt.

Synthesis of 1,10-phenanthroline-5,6-dione (phendione):$^{12}$

4 g (20 mmol) 1,10-phenanthroline and 3.6 g (30 mmol) KBr was added to a 125 mL round bottom flask and chilled in an ice bath. 1.5:1 mixture of concentrated H$_2$SO$_4$: HNO$_3$(40/20 mL respectively) were added dropwise to the flask. Caution: The reaction is very exothermic and evolves bromine gas. The reaction should be carried out in the hood with the hood sash at a minimum height. Care should be taken handling the concentrated acids. Following addition of the acid mixture, the solution was refluxed for 3 hours at 100 °C. The solution was removed from heat and allowed to cool to room temperature. The bright orange solution was poured over 500 mL of ice in a large beaker and the flask was rinsed with ice and was slowly neutralized with NaOH pellets producing a milky, dark yellow solution. Care should be taken to not overshoot the neutral pH producing a basic solution. At basic pH’s, the solution turns green and result in decreased yields. The product was extracted in CHCL$_3$ and dried over magnesium sulfate producing a clear yellow solution that was concentrated using rotary evaporation and dried under vacuum. The methylated analogue 2,9-dimethyl-1,10-phenanthroline-5,6-dione (dmphendione) was prepared in a similar method using the 2,9-dimethyl-1,10-phenanthroline (dmphen) starting material.
Synthesis of dipyrido[3,2-f:2',3'-h-quinoxaline (dpq):\textsuperscript{13}

\textit{Caution: Phendione is a flocculent solid and is a mucous irritant. To reduce exposure, tare the reaction flask and transfer the material in the fume hood.} 1 g (47.5 mmol) phendione and 350 mL ethanol were added to a 500 mL round bottom flask. 0.49 mL (d=0.899 gcm\(^{-3}\), 71 mmol) 1,2-diaminoethane was added to the flask (solution golden brown) and heated at 40 \(^\circ\)C for 2 hours. The reaction was monitored by TLC using 10% methanol in methylene chloride. The reaction was removed from heat and allowed to stir for 5 hours at room temperature producing a golden, brown solution. The solution was reduced by rotary evaporation to yield a white solid that was recrystallized from boiling methanol to yield an off-white solid. The dimethyl analogue of this ligand was prepared in a similar method using the dmphendione starting material with similar yields.

Synthesis of dipyrido[3,2-~:2',3'-c]phenazine (dppz):\textsuperscript{13-14}

1 g (47.5 mmol) phendione and 0.77 g (71 mmol) 1,2-phenylene-diamine were added to a 120mL pressure tube with 1:2 EtOH: dH\(_2\)O (30 mL: 60 mL). The pressure tube was placed in an oil bath at 180\(^\circ\)C and stirred for 3 hours producing a brownish-orange solution. \textit{Caution: A blast shield should be utilized with the hood sash at minimum height to reduce explosion hazards.} The reaction tube was removed from heat and allowed to cool producing a feathery, yellow solid that was collected by vacuum filtration. Additional crops of precipitate were obtained upon cooling the mother liquor and were collected by vacuum filtration. Similar TLC conditions used in the synthesis of the dpq ligand were used to monitor the reaction. The dimethyl
analogue (dmdppz) of this ligand was prepared in a similar method using the
dmphendione starting material with similar yields.

Spectroscopic Characterization of Synthesized Ligands:

1,10-phenanthroline-5,6-dione (phendione):

Yield: 3.8 g (90%). $^1$H NMR (CDCl$_3$, 400 MHz): $^1$H NMR (CDCl$_3$): $\delta$ 9.05 (d, $J=4.76$ Hz, 2H), 8.43 (d, $J=7.51$ Hz, 2H), 7.53 (t, $J=6.23$, 2H).

2,9-dimethyl-1,10-phenanthroline-5,6-dione (dmphendione):

Yield: 1.9 g (75%). $^1$H NMR (CDCl$_3$, 400 MHz): $^1$H NMR (CDCl$_3$): $\delta$ 8.36 (d, $J=8.06$ Hz, 2H), 7.40 (d, $J=7.76$ Hz, 2H), 2.83 (s, 3H).

Dipyrido[3,2-f:2',3'-h]quinoxaline (dpq):

Yield: 1.03 g (92.8%). $^1$H NMR (CDCl$_3$, 400 MHz): $^1$H NMR (CDCl$_3$): $\delta$ 9.47 (d, $J=8.24$ Hz, 2H), 9.27 (d, $J=4.40$ Hz, 2H), 8.97 (s, 2H), 7.78 (td, 6.22 Hz, 4.40 Hz, 2H).

Dimethyl-dipyrido[3,2-f:2',3'-h]quinoxaline (dmdpq):

Yield: 1.1 g (88%). $^1$H NMR (CDCl$_3$, 400 MHz): $^1$H NMR (CDCl$_3$): $\delta$ 9.37 (d, $J=8.24$ Hz, 2H), 8.93 (s, 2H), 7.65 (d, $J=8.42$ Hz, 2H), 3.07 (s, 6H).

Dipyrido[3,2-f:2',3'-c]phenazine (dppz):

Yield: 872 mg (65%). $^1$H NMR (CDCl$_3$, 400 MHz): $^1$H NMR (CDCl$_3$): $\delta$ 9.17 (d, $J=8.15$ Hz, 2H), 8.90 (d, $J=4.49$ Hz, 2H), 8.01 (d, $J=6.59$ Hz, 2H), 7.78 (d, 6.50 Hz, 2H), 7.61 (td, $J=6.23$ Hz, 6.41 Hz, 2H). ESI MS calcd for C$_{18}$H$_{10}$N$_4$[M]$^+$ 282.1, found 283 [M]$^+$. 

23
Dimethyl-dipyrid0[3,2-~:2',3'-c]phenazine (dmdppz):

Yield: 0.9 g (65%). $^1$H NMR (CDCl$_3$, 400 MHz): $^1$H NMR (CDCl$_3$): $\delta$9.50 (d, $J$=8.24 Hz, 2H), 8.31 (d, $J$=6.59 Hz, 2H), 7.81 (d, $J$=6.50 Hz, 2H), 7.63 (d, $J$=8.24 Hz, 2H), 2.97 (s, 6H). ESI MS calcd for C$_{20}$H$_{14}$N$_4$[M]$^+$310.12, found 311 [M]$^+$.

Spectroscopic Characterization of Synthesized Ruthenium Complexes:

Complexes GL005, GL008, and GL013 are sulfonate or carboxylate containing complexes that could not be isolated from water due to solubility. Because of this, NMR data could not be obtained. ESI-MS data are given for each complex.

Ru(bpy)$_2$dmbpy, GL002:

Yield: 297 mg (87%). $^1$H NMR (CD$_3$CN, 400 MHz): $\delta$ 8.48 (d, $J$=8.24 Hz, 2H), 8.40 (d, $J$=8.42 Hz, 2H), 8.27 (d, $J$=8.42 Hz, 2H), 8.08 (td, $J$=8.06, 1.65 Hz, 2H), 7.96-7.90 (m, 6H), 7.62 (d, $J$=5.31 Hz, 2H), 7.46 (td, $J$=5.86, 1.65 Hz, 2H), 7.29 (d, $J$=6.59 Hz, 2H), 7.21 (td, $J$=7.32, 1.47 Hz, 2H), 2.14 (s, 6H). $^{13}$C NMR (CD$_3$CN): $\delta$ 166.69, 159.91, 158.68, 158.44, 154.06, 152.90, 139.22, 138.85, 138.81, 129.18, 128.49, 128.29, 125.47, 125.39, 123.18, 25.62. ESI MS calcd for C$_{32}$H$_{28}$N$_6$Ru [M]$^+$ 598.1, [M]$^{2+}$ 299; found 598.3 [M]$^+$, 299.1 [M]$^{2+}$. Purity by HPLC: 98.8% by area. UV/Vis (MeCN) $\epsilon$: 247 nm (23100), 289 (72500), 452 (13900).

Ru(bpy)$_2$dmdpq, GL003:

Yield: 284 mg (77%). $^1$H NMR (CD$_3$CN, 400 MHz): $\delta$ 9.51 (d, $J$ = 8.4 Hz, 2H), 9.17 (s, 2H), 8.5 (dd, $J$ = 8.24, 8.24 Hz, 4H), 8.02 (quin, $J$ = 7.69, 4H), 7.72-7.80 (m, 6H), 7.29
(t, J = 6.68, 4H), 2.19 (s, 6H). $^{13}$C NMR (CD$_3$CN): δ 169.63, 158.65, 158.52, 154.14, 152.98, 151.44, 147.69, 139.84, 139.05, 138.84, 134.96, 129.47 129.04, 128.53, 128.49, 125.62, 125.54, 26.51.  ESI MS calcd for C$_{36}$H$_{28}$N$_8$Ru [M]$^+$ 674.1, [M]$^{2+}$ 337; found 673.1 [M]$^+$, 336.9 [M]$^{2+}$. Purity by HPLC: 95.1% by area. UV/Vis (MeCN) ε: 256 nm (49700), 289 (65100), 452 (13100).

Ru(bpy)$_2$phen, GL004:

Prepared per literature procedure.$^{15}$ $^1$H NMR (CD$_3$CN, 400 MHz): δ 8.41 (d, $J = 8.02$ Hz, 2H), 8.36 (d, $J = 8.2$ Hz, 2H), 8.01 (s, 2H), 7.91 (t, $J = 7.8$ Hz, 2H), 7.78 (dt, $J = 10.7$ Hz, 5.3 Hz, 4H), 7.53 (t, $J = 6.60$, 2H), 7.45 (d, $J = 5.0$, 2H), 7.25 (t, $J = 6.3$, 2H), 7.01 (t, $J = 6.25$, 2H). $^{13}$C NMR (CD$_3$CN): δ 157.23, 157.02, 151.96, 151.65, 151.61, 147.49, 137.47, 137.36, 136.55, 130.75, 127.81, 126.97, 126.93, 126.93, 126.80, 125.46, 123.88, 123.82. Additional $^{13}$C peaks due to concentration dependent molecular aggregation through π-stacking in solution.$^{16}$ ESI MS calcd for C$_{32}$H$_{24}$N$_6$Ru [M]$^{2+}$ 287.01, found 287 [M]$^{2+}$. Purity by HPLC: 98.1% by area.

Ru(bpds)$_2$dmmbpy, GL005:

ESI MS calcd for C$_{60}$H$_{40}$N$_6$O$_{12}$RuS$_4$ [M]$^{2-}$633.03, found 632.6 [M]$^{2+}$.

Yield: 150 mg (90%). $^1$H NMR (CD$_3$CN, 400 MHz): δ 8.50 (d, $J = 7.6$ Hz, 4H), 8.05 (m, 4H), 7.83 (d, $J = 7.8$ Hz, 4H), 7.70 (d, $J = 5.6$ Hz, 2H), 7.59 (d, $J = 5.1$ Hz, 2H), 7.39 (m, 4H), 7.26 (s, 2H), 2.47 (s, 6H). $^{13}$C NMR (CD$_3$CN): δ 148.28, 158.159, 158.053, 153.081, 150.606, 141.89, 138.71, 138.67, 137.87, 128.50, 128.34, 126.80, 125.28, 125.19,
21.38. ESI MS calcd for $\text{C}_{32}\text{H}_{28}\text{N}_6\text{Ru} \ [\text{M}]^{2+}$ 299.07, found 299.1 [M]$^{2+}$. Purity by HPLC: 98.8% by area. UV/Vis (MeCN) $\varepsilon$: 240 nm (26500), 289 (71600), 453 (13500)

Ru(bpy)$_2$dmphen, GL007

Yield: 160 mg (95%). $^1$H NMR (CD$_3$CN, 400 MHz): $^1$H NMR (CD$_3$CN): $\delta$ 8.57 (d, $J = 8.0$ Hz, 2H), 8.50 (d, $J = 7.8$ Hz, 2H), 8.09 (dt, $J = 8.0$, 1.4 Hz, 2H), 8.05 (s, 2H), 8.02 (td, $J = 8.2$, 1.5 Hz, 2H), 7.82 (d, $J = 5.8$ Hz, 2H), 7.77 (d, $J = 5.8$ Hz, 2H), 7.78-5.59 (m, 4H), 7.34 (td, $J = 9.0$, 1.4 Hz, 2H), 7.31 (td, $J = 9.0$, 1.4 Hz, 2H), 2.0 (s, 6H). $^{13}$C NMR (CD$_3$CN): $\delta$ 167.50, 158.75, 158.4, 1554.01, 153.96, 152.82, 152.86, 150.53, 150.34, 138.90, 138.74, 136.87, 130.79, 130.62, 130.14, 128.94, 1218.86, 128.53, 128.48, 128.41, 128.34, 125.87, 125.59, 125.49, 26.49.$^{16}$ESI MS calcd for $\text{C}_{34}\text{H}_{28}\text{N}_6\text{Ru} \ [\text{M}]^{*}$ +622.14, found [M]$^{*}$+621.2, [M]$^{2+}$311.1. Purity by HPLC: 99.9% by area. UV/Vis (MeCN) $\varepsilon$: 287 nm (60200), 452 (12800).

Ru(bpy)$_2$bcds, GL008:

ESI MS calcd for $\text{C}_{46}\text{H}_{34}\text{N}_6\text{O}_6\text{RuS}_2 \ [\text{M}]^{+}$ 932.10, [M]$^{2+}$466.1 found [M]$^{+}$ 955.3 (+Na), [M]$^{2+}$ 488.9 (+Na).

Ru(bpy)$_2$dppz, GL009:

Yield: 133 mg (35%). $^1$H NMR (CD$_3$CN, 400 MHz): $^1$H NMR (CD$_3$CN): $\delta$ 9.32 (dd, $J = 8.24$, 1.5 Hz, 2H), 8.60 (t, $J = 9.18$ Hz, 4H), 8.20-8.13 (m, 6H), 8.06 (t, $J = 7.69$ Hz, 2H), 7.98-7.91 (m, 4H), 7.84-7.77 (m, 4H), 7.52 (t, $J = 6.32$ Hz, 2H), 7.36 (t, $J = 6.87$ Hz, 2H). $^{13}$C NMR (CD$_3$CN): $\delta$ 158.27, 158.07, 154.77, 153.20, 153.06, 151.56, 143.84, 141.12, 139.06, 138.97, 134.58, 133.62, 1311.95, 130.71, 128.70, 128.55, 128.50, 125.41,
125.35. ESI MS calcd for C_{38}H_{26}N_{8}Ru [M]+ 696.13, [M]^{2+} 348.1 found [M]+ 841.1 (PF_6),
[M]^{2+} 348.0. Purity by HPLC: 98.7% by area. UV/Vis (MeCN) ε: 255 nm (40600), 285
(85000), 367 (16400), 448 (16000).

Ru(bpy)$_2$dmppz, GL010:

Yield: 133 mg (35%). $^1$H NMR (CD$_3$CN, 400 MHz): δ 9.78 (d, J = 8.2 Hz, 2H), 8.53 (d, J =
8.0 Hz, 2H), 8.47 (d, J = 8.2 Hz, 2H), 8.41-8.39 (m, 2H), 8.09-7.99 (m, 4H), 7.86 (d, J =
5.1 Hz, 2H), 7.79-7.72 (m, 4H), 7.34-7.28 (m, 4H), 2.16, (s, 6H). $^{13}$C NMR (CD$_3$CN): δ
169.73, 158.67, 158.50, 154.12, 152.99, 152.67, 143.97, 140.32, 139.08, 138.89,
135.25, 133.23, 130.57, 129.63, 129.50, 128.60, 128.50, 125.66, 125.55, 26.48. ESI
MS calcd for C$_{40}$H$_{30}$N$_{8}$Ru [M]+ 724.16, [M]^{2+} 362.1 found [M]+ 723.3, [M]^{2+} 362.1. Purity
by HPLC: 97.4% by area. UV/Vis (MeCN) ε: 284 nm (88900), 325 (23600), 352
(18900), 450 (14100).

Ru(bpy)$_2$biq, GL011:

Yield: 772.4 mg (99%). $^1$H NMR (CD$_3$CN, 400 MHz): δ 88.96 (d, J = 8.8 Hz, 2H), 8.78 (dd,
J = 8.4, 1.1 Hz, 2H), 8.72 (d, J = 8.8 Hz, 2H), 8.65 (dd, J = 8.2, 1.1 Hz, 2H), 8.39 (dd, J =
5.3, 1.1 Hz, 2H), 8.29 (q, J = 8.9 Hz, 4H), 8.01-7.98 (m, 4H), 7.91 (d, J = 5.3 Hz, 1H),
7.89 (d, J = 5.3 Hz, 1H), 7.64 (d, J = 5.5 Hz, 2H), 7.62 (d, J = 5.5 Hz, 1H), 7.47 (td, J =
7.9, 0.9 Hz, 2H), 7.11 (d, J = 8.8 Hz, 2H), 7.05-7.01 (m, 2H). $^{13}$C NMR (CD$_3$CN): δ
161.95, 1588.69, 154.08, 152.15, 149.24, 148.54, 140.36, 138.49, 138.29, 132.36,
132.14, 132.00, 130.57, 130.10, 1129.98, 129.27, 129.14, 127.22, 126.89, 125.73,
122.08. ESI MS calcd for C$_{42}$H$_{28}$N$_{6}$Ru [M]+ 718.14, [M]^{2+} 359.1, found [M]+ 718.3, [M].
2+359. Purity by HPLC: 99.9 % by area.UV/Vis (MeCN) ε: 218 nm (78000), 338 (28400), 378 (19300), 440 (8300), 525 (8300).

Ru(bpy)$_2$·2,2’-biq·4,4’-dca, GL013


Ru(bpy)$_2$bathocuprione, GL014

Yield: 607.8 mg (98.2 %). $^1$H NMR (CD$_3$CN, 400 MHz): δ 8.61 (d, $J = 8.0$ Hz, 2H), 8.54 (d, $J = 8.2$ Hz, 2H), 8.10 (td, $J = 7.9, 1.1$ Hz, 2H), 8.04-7.98 (m, 4H), 7.88 (d, $J = 5.5$ Hz, 2H), 7.82 (d, $J = 5.3$ Hz, 2H), 7.61-7.52 (m, 12H), 7.42 (t, $J = 7.0$ Hz, 2H), 7.34 (t, $J = 6.9$ Hz, 2H), 2.01 (s, 6H). $^{13}$C NMR (CD$_3$CN): δ 167.48, 158.73, 158.47, 153.96, 152.86, 150.51, 150.33, 138.89, 138.72, 136.85, 130.77, 130.62, 130.12, 128.88, 128.51, 128.43, 128.35, 125.86, 125.56, 125.47, 26.47. $^{16}$ESI MS calcd for C$_{46}$H$_{36}$N$_6$Ru [M]$^+$774.2, [M]$^{2+}$ 387.1, found [M]$^+$773.3, [M]$^{2+}$387.1. Purity by HPLC: 95.0% by area.UV/Vis (MeCN) ε:290 (74100), 454 (15600).

Ru(dmphen)$_2$·8HQ, GL018:

Yield: 55 mg (24%). $^1$H NMR (CD$_3$CN, 400 MHz): δ 8.51-8.44 (m, 2H), 8.30 (d, $J = 8.4$ Hz, 1H), 8.19-7.97 (m, 5H), 7.81-7.74 (m, 1H), 7.69 (d, $J = 8.2$ Hz, 1H), 7.61 (J = 8.2 Hz), 7.35 (dd, $J = 8.4, 2.9$ Hz, 2H), 7.09 (t, $J = 8.0$ Hz, 1H), 6.72-6.64 (m, 3H), 6.25 (d, $J = 8.0$ Hz, 1H). $^{13}$C NMR (CD$_3$CN): δ 169.81, 169.31, 169.00, 168.78, 167.87, 167.53, 167.43, 166.80, 160.28, 160.27, 152.13, 152.05, 151.35, 151.19, 149.95, 149.53, 149.02, 147.06, 136.59, 136.47, 136.43, 135.95, 135.52, 130.49, 130.38, 130.30, 129.99, 127.81, 127.59, 127.52, 127.41, 126.10, 126.07, 26.311, 26.27, 25.04, 24.57.
ESI MS calcd for C_{37}H_{30}N_{5}ORu [M]+662.15, found [M]+662.1. Purity by HPLC: 99.9% by area. UV/Vis (dH_{2}O) ε: 224 (64700), 268 (52000), 450 (8100), 497 (8800), 620 (9900).

Ru(dmphen)_{2}-2Me8HQ, GL019:

Yield: 96 mg (58%). ¹H NMR (CD_{3}CN, 400 MHz): δ 8.40-8.36 (m, 3H), 8.29 (d, J = 8.4 Hz, 1H), 8.23 (d, J = 8.2 Hz, 1H), 8.10-8.02 (m, 5H), 7.88 (s, 1H), 7.75 (d, J = 8.6 Hz, 1H), 7.69-7.64 (m, 2H), 7.44 (d, J = 8.2 Hz, 1H), 7.38 (d, J = 8.2 Hz, 2H), 7.00 (t, J = 7.8 Hz, 1H), 6.66 (d, J = 8.6 Hz, 1H), 6.60 (d, J = 7.8 Hz, 1H), 6.16 (d, J = 7.8 Hz, 1H), 2.24 (s, 3H). ¹³C NMR (CD_{3}CN, 400 MHz): δ 170.51, 168.99, 168.93, 168.76, 166.66, 161.79, 160.31, 160.28, 160.25, 160.24, 154.79, 151.79, 151.72, 146.52, 137.94, 136.66, 136.55, 136.21, 135.77, 130.33, 130.21, 129.97, 129.90, 129.85, 129.13, 127.72, 127.7, 127.60, 127.36, 127.20, 127.13, 126.81, 126.64, 26.38, 24.67, 24.66, 24.03. ESI MS calcd for C_{38}H_{32}N_{5}ORu [M]+676.17, found [M]+676.1. Purity by HPLC: 97.0% by area. UV/Vis (MeCN) ε: 225 nm (62500), 272 (56400), 500 (8800).

Ru(bpy)_{2}dpq, GL021:

Yield: 237 mg (66%). ¹H NMR (CD_{3}CN, 400 MHz): δ 9.55 (d, J = 8.2 Hz, 2H), 9.23 (s, J = 0.6 Hz, 2H), 8.56 (dd, J = 7.8, 7.8 Hz, 6H), 8.22 (dd, J = 5.3, 0.6 Hz, 2H), 8.14 (t, J = 7.8 Hz, 2H), 8.03 (t, J = 7.6 Hz, 2H), 7.92-7.87 (m, 4H), 7.68 (d, J = 5.6 Hz, 2H). ¹³C NMR (CD_{3}CN): δ 158.86, 158.61, 155.34, 153.75, 153.70, 153.64, 153.61, 150.89, 147.37, 141.45, 139.60, 139.49, 134.93, 131.83, 129.27, 129.21, 129.11, 129.06, 128.74, 125.96, 125.89. ESI MS calcd for C_{34}H_{32}N_{8}Ru [M]+646.12, found [M]+645.9.
Purity by HPLC: 95.4% by area. UV/Vis (MeCN) ε: 256 nm (53600), 289 (55900), 449 (15100).

Ru(dmphen)₂bpy, GL022:

Yield: 98 mg (20%). ¹H NMR (CD₃CN, 400 MHz): δ 8.66 (d, J = 8.24 Hz, 2H), 8.33 (d, J = 8.42 Hz, 2H), 8.25 (d, J = 879 Hz, 2H), 8.18 (d, J = 8.24 Hz, 2H), 8.11 (d, J = 8.42 Hz, 2H), 7.83-7.75 (m, 4H) 7.41 (d, J = 8.2 Hz, 2H), 7.03 (d, J = 5.86 Hz, 2H), 6.99-6.95 (m, 2H), 2.16 (s, 12H). ¹³C NMR (CD₃CN): δ 169.68, 167.74, 158.96, 153.04, 150.42, 149.39, 138.98, 138.86, 137.82, 131.06, 130.96, 128.51, 128.37, 128.23, 127.96, 127.62, 124.90, 26.85, 25.77. ESI MS calcd for C₃₈H₃₂N₆Ru [M]+674.17, [M]²⁺ 337.1, found [M]+673.2, [M]²⁺337.0. Purity by HPLC: 94.0% by area. UV/Vis (MeCN) ε: 270 nm (52100), 290 (29500), 457 (9000).

Ru(dmphen)₂bathophen, GL023:

Yield: 101.8 mg (49%). ¹H NMR (CD₃CN, 400 MHz): δ 8.75 (d, J = 8.4 Hz, 2H), 8.30 (d, J = 8.0 Hz, 4H), 8.15 (d, J = 8.8 Hz, 2H), 7.99 (s, 2H), 8.36 (d, J = 8.4 Hz, 2H), 7.56-7.55 (m, 6H), 7.46-7.44 (m, 6H), 7.32-7.28 (m, 4H), 2.02 (s, 12H). ¹³C NMR (CD₃CN): δ 168.72, 166.96, 152.53, 149.46, 149.19, 148.95, 148.32, 137.96, 136.74, 135.29, 130.19, 129.98, 129.74, 129.72, 129.06, 128.30, 127.43, 127.31, 127.28, 126.59, 125.89, 125.27, 26.14, 24.84. ESI MS calcd for C₅₂H₄₁N₆Ru [M]+851.24, found [M]+424.8. Purity by HPLC: 99.9% by area. UV/Vis (MeCN) ε: 220 nm (82300), 270 (80100), 470 (16700).

Ru(dmphen)₂dqp, GL039:
Yield: 101.8 mg (49 %). $^1$H NMR (CD$_3$CN, 400 MHz): $\delta$ 8.73 (d, $J = 8.4$ Hz, 2H), 8.42-8.39 (m, 2H), 8.31-8.24 (m, 5H) 8.12 (d, $J = 8.8$ Hz, 2H), 8.06-8.05 (m, 2H), 7.83 (d, $J = 8.4$ Hz, 2H), 7.38-7.33 (m, 5H), 7.22 (d, $J = 8.2$ Hz, 2H), 2.16 (s, 12H). $^{13}$C NMR (CD$_3$CN): $\delta$ 169.81, 167.92, 154.09, 140.52, 149.37, 149.34, 139.01, 138.20, 137.63, 131.00, 129.01, 128.51, 128.29, 128.23, 127.52, 126.17, 26.88, 25.88. $^{16}$ESI MS calcd for C$_{42}$H$_{432}$N$_8$Ru [M]+750.18, [M]$^{2+}$ 375.1 found [M]+749.8, [M]$^{2+}$375.0. Purity by HPLC: 98.1% by area. UV/Vis (MeCN) $\varepsilon$: 224 nm (79900), 268 (79400), 459 (14900).
Table 2.1: Ligand structures used in complexes with corresponding acronyms

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<th>Acronym</th>
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<td><img src="2bipyridine.png" alt="Image" /></td>
<td>2,2'-bipyridine</td>
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Table 2.1 cont): Ligand structures used in complexes with corresponding acronyms

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Table 2.1 cont): Ligand structures used in complexes with corresponding acronyms

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Table 2.1 cont): Ligand structures used in complexes with corresponding acronyms

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<td><img src="image4" alt="Complex Structure 4" /></td>
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Purity of Synthesized Complexes by HPLC

Chloride salts of the ruthenium complexes were injected on an Agilent 1100 Series HPLC equipped with a model G1311A quaternary pump, G1315B UV diode array detector and Chemstation software version B.01.03. Chromatographic conditions were optimized on a Column Technologies Inc. C18, 120 Å (250 mm x 4.6 mm inner diameter, 5 µm) fitted with a Phenomenex C18 (4 mm x 3 mm) guard column. The detection wavelength was 280 nm. Mobile phases used were 0.1% formic acid in dH₂O and 0.1% formic acid in HPLC grade acetonitrile. See table below for gradient used.

Table 2.3: HPLC gradient used to test purity of synthesized ruthenium complexes

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<th>Time (minutes)</th>
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<th>0.1% formic in MeCN</th>
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Chapter 3. Photochemistry of Ruthenium Complexes: Characterization by UV-Vis Spectroscopy and Mass Spectrometry

1. Introduction

Successful PDT complexes should efficiently produce the desired active species upon light activation, but not react so quickly under ambient light conditions as to inhibit administration of the drug. Moderate light stability is required to ensure that the product can be efficiently handled and transferred by medical personnel to the patient, and upon light activation, provide the desired cytotoxic product. However, the complexes have to react sufficiently quickly for the patient to get an appropriate light dose in a short time to be medically useful. Photoejection experiments of the complexes were monitored by UV-Vis to observe the rate of conversion of ruthenium complex reactants to products. Mass spectrometry was used to characterize the photochemical products of the reactions. This provides the identity of the active species that is responsible for the biological effects observed in Chapters 4 and 5.

Ruthenium complexes that are not sterically strained do not exhibit photodegradation due to the low efficiency of population of the $^3\text{MC}$ excited state after photoexcitation at room or physiological temperatures (22 or 37 °C). These compounds are stable in the presence of light. Several unstrained complexes were synthesized to act as control compounds. Alternatively, several strained complexes have been synthesized that are capable of populating the $^3\text{MC}$ state, resulting in ligand ejection upon light activation (see Chapter 2.2).
2 Photoejection Experiments Monitored by UV-Vis Spectroscopy

UV-Vis spectroscopy was used to observe the absorbance properties of the synthesized ruthenium complexes. Due to the number of complexes analyzed for this study, all absorption profile figures are included at the end of the chapter.

Figure 3.1 shows the typical absorbance spectra of ruthenium complexes that are stable in the presence of light. Complexes GL004, 009, and 021 display an absorbance maximum at ~450 nm, and the absorption spectra do not change upon exposure to light.

In contrast, sterically hindered, octahedral ruthenium complexes can successfully shuttle electrons into the $^3$MC excited state with blue light activation (see Figure 2.2). Exposure to blue light was punctuated by periodic UV-Vis scans in order to track the conversion of the starting complex to the photoproduct. The difference in absorbance at ~495 nm vs ~410 nm ($\Delta A_{495-410}$) were plotted against time (minutes) to show the time dependence for the photochemical reactions. Curves were fit and kinetic half-lives were determined with a ‘one phase association’ equation to yield the calculated half-life ($t_{1/2}$) of the complex using Prism software. Spectral changes observed for photo-ejecting complexes are displayed in Figure 3.2 and $t_{1/2}$ values are summarized in Table 3.1.

GL002 displays a good balance of light activated activity, with a near-optimal half-life of 1.9 minutes observed for the complex. The complex employs the Ru(bpy)$_2$ backbone and ejects the strained 6,6′-dimethyl-2,2′-bipyridine ligand (see
Table 3.2). Structural differences in the sterically strained ligand produce different ejection profiles. For example, GL007 has a half-life of 112.8 minutes, ~60 times slower than GL002 (see Table 3.1). GL007 does not incorporate the 6,6'-dimethyl-2,2'-bipyridine ligand; instead, it contains 2,9-dimethyl-1,10-phenanthroline (see Table 2.1). An apparent trend is that complexes utilizing the 6,6'-dimethyl-2,2'-bipyridine ligand tend to degrade faster than fused ring systems such as 1,10-phenanthroline. Increasing the planar surface in the fused ligands by adding a dmdpq (complex GL003) or dmdppz (complex GL010) ligand (see Table 2.1) to the Ru(bpy)₂ scaffold further supports this observation. GL003 has a long half-life of 60.9 minutes, and GL010 has a half-life greater than 6 hours. In order to obtain an ejection profile for GL010, intense white light was employed instead of blue light used in other studies. Under these conditions, the half-life of the complex is 20.5 minutes (see Table 3.1).

The differences in photoreaction half-lives provide useful guidelines for the development of these complexes, and it is clear that strained bipyridine ligands eject faster than fused ring systems incorporated in complexes such as GL003, 007, 010 (see Table 3.3). Recoordination of more rigid ligands such as dmphen, dmdpq, and dmdppz (see Table 2.1) appears to occur readily, hindering the complete photo-dissociation of the ligand and resulting in longer half lives (see Table 3.1). The enhanced photoejection rate for complexes of 6,6'-dimethyl-2,2'-bipyridine is thought to be due to the ability of the ligand to rotate freely around the C²-C² bond, thwarting recoordination and resulting in ligand loss.
The half-lives of the ruthenium complexes are summarized in Table 3.1. The fastest ejecting complexes are the ones utilizing the 2,2’-6,6’-dimethyl-bipyridine ligand. Complex GL002 and 005 have half-lives of 1.9 and 0.6 minutes respectively. Moving the methyl group around the bipyridine ligand to the 3,3’ positions provides a different type of steric clash (“backside clash”) seen in complex GL006, significantly increasing the t_{1/2} to 230 minutes.

The ejection kinetics for GL002 are ~50 fold faster than GL011, a complex containing a biquinoline ligand. It was thought that the addition of a phenyl ring to the 2,2-bipyridine motif to make the biquinoline ligand would provide more steric clash and thus a faster photoactive complex. This was not observed, and is thought that the size of the six methyl hydrogens provides more steric clash compared to the bulk of the biquinoline ligand with two benzyl hydrogens.

To observe the effect of electron withdrawing groups on ejection kinetics, GL008 and 014 were synthesized. Both compounds contain the same Ru(bpy)_2 backbone and only differ by the addition of sulfonate groups to complex GL008 (see Table 2.2). A six-fold increase in the ejection kinetics was observed for GL008 and 014, with t_{1/2} values of 12.6 and 59.7 minutes respectively (see Table 3.1). The addition of these groups may have a negative effect on the ability of the nitrogen atoms to efficiently donate their electrons to the ruthenium center and could be another mechanism by which the ejection kinetics can be tuned.

However, addition of electron withdrawing carboxylic acid groups to the biquinoline ligand appear to have the opposite effect when comparing complexes

45
GL011 and 013 (see Table 2.2). Complex GL013, contains carboxylic acid groups at the 4,4’ positions and displays the slowest ejection kinetics observed under blue light, with a t\(_{1/2}\) of greater than six hours (see Table 3.1). Intense white light was also used on this complex to get an ejection profile in a timely manner. Under intense white light, the observed t\(_{1/2}\) was 242.3 minutes. Reduction of the electron donating ability of the nitrogen atoms through the addition of electron withdrawing carboxylic groups is not observed in complex GL013. Additional complexes with the same bis-1,10-phenanthroline backbone containing ligands such as 6,6’-dimethyl-2,2’-bipyridine with carboxylic acid groups at the 4,4’ position should be synthesized to understand why complex GL011 has faster ejection kinetics than GL013.

The addition of 4,7-diphenyl groups to the 1,10-phenanthroline ligand also appears to hinder dissociation. A three fold kinetic difference (5.4 and 15.2 minutes respectively) is observed in the half-lives of GL022 and 023. It is possible that the addition of the para-electron donating phenyl groups stabilize the nitrogen-ruthenium bond, hindering photo-dissociation. The 6,6-dimethyl-4,7-diphenyl-2,2’-bipyridine analogue containing complexes that contain the electron donating phenyl groups at the meta and ortho positions could be synthesized to observe how the position of the electron withdrawing groups affects the kinetic activity.

Complexes containing different backbones have also been prepared. GL018, 019, 022, and 023 contain the Ru(dmphen)\(_2\) backbone, and have different ejection profiles. The quinoline containing compounds GL018 and 019 display different
kinetics, even though the only difference between the complexes is the addition of a methyl group at the two-position on the quinoline ligand. Modeling may be necessary to understand why GL018 ejects faster, and produces a mix of products (see Table 3.2) as opposed to the more sterically hindered GL019, which ejects slower and produces one photo-dissociation product.

Very reactive complexes containing two sterically hindered ligands such as the Ru(dmbpy)_2 starting material with 2,2’-bipyridine as well as the slower ejecting dmpdq ligand were synthesized. Products of these reactions were confirmed by the absence of an emissive spot on thin layer chromatography and by mass spectrometry of the reaction mixture, but the compounds readily degraded upon flash chromatography purification and were unable to be characterized. This showed that the Ru(dmbpy)_2 backbone is too reactive for the generation of useful complexes for PDT applications.

In order to tune the absorption profiles of the metal complexes, a synthetic approach was attempted to generate complexes that absorb closer to the infrared region of the spectrum. Compounds that readily degrade to their corresponding activated species utilizing longer wavelength light are desirable as the longer wavelength light is capable of penetrating deeper tissues. The Ru(phen)_2Cl_2 scaffold coupled with the biquinoline ligand provides an example of the red shift sought (GL011, Figure 3.2). The biquinoline ligand was selected for its extended pi system, as it should reduce the MLCT band energy (see Figure 2.2). As expected, a red shift in absorbance maxima from the typical 450 nm to 510 nm is observed in complex
GL011. The strain from the additional ring on the biquinoline ligand produces a photoejecting complex (see Table 3.2).

The desired red shift was also observed utilizing the cyclometallated ligands 2-phenylpyridine and 7,8-benzoquinoline coupled with the Ru(bpy)$_2$ backbone shown in complexes GL034 and 035 (Figure 3.1) Cyclometallated systems GL034 and 035 are significantly red shifted, as expected, with observed absorbance maxima centered at 550 nm. However, these compounds do not eject as they are unstrained and are unable to populate the $^3$MC excited state. As a result, no spectral change is observed upon exposure to light. Synthesis of cyclometallated complexes with the strained Ru(dmphen)$_2$ backbone should be performed to obtain the desired red shift while producing a photo-active species capable of photo-binding DNA.
Figure 3.1: Non-photoejecting Complexes

GL004

GL009

GL024
Figure 3.1: Non-photoejecting Complexes (cont.)

GL034

GL035
Figure 3.2: Photo-ejecting Complexes

GL002

GL003

GL005

GL006

\[ t_{1/2} = 1.9 \text{ min} \]

\[ t_{1/2} = 60.9 \text{ min} \]

\[ t_{1/2} = 0.6 \text{ min} \]

\[ t_{1/2} = 230 \text{ min} \]
Figure 3.2: Photo-ejecting Complexes (cont.)

GL007

GL008

GL010 – white light curve shown

GL011
Figure 3.2: Photo-ejecting Complexes (cont.)

GL013

GL014

GL018

GL019
Figure 3.2: Photo-ejecting Complexes (cont.)

GL022

GL023

GL039
Table 3.1: Complex $t_{1/2}$ values

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>Complex</th>
<th>$t_{1/2}$ (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL002</td>
<td>Ru(bpy)$_2$dmmbpy</td>
<td>1.9</td>
</tr>
<tr>
<td>GL003</td>
<td>Ru(bpy)$_2$mdpq</td>
<td>60.9</td>
</tr>
<tr>
<td>GL005</td>
<td>Ru(bpds)$_2$dmmbpy</td>
<td>0.6</td>
</tr>
<tr>
<td>GL006</td>
<td>Ru(bpy)$_2$-3,3’dmbpy</td>
<td>230</td>
</tr>
<tr>
<td>GL007</td>
<td>Ru(bpy)$_2$dmphen</td>
<td>112.8</td>
</tr>
<tr>
<td>GL008</td>
<td>Ru(bpy)$_2$bcds</td>
<td>12.6</td>
</tr>
<tr>
<td>GL010</td>
<td>Ru(bpy)$_2$mdmppz</td>
<td>&gt;6hrs/20.5*</td>
</tr>
<tr>
<td>GL011</td>
<td>Ru(phen)$_2$biq</td>
<td>89.2</td>
</tr>
<tr>
<td>GL013</td>
<td>Ru(bpy)$_2$-2,2’biq-4,4’-dca</td>
<td>&gt;8hrs/242.3*</td>
</tr>
<tr>
<td>GL014</td>
<td>Ru(bpy)$_2$bc</td>
<td>59.7</td>
</tr>
<tr>
<td>GL018</td>
<td>Ru(dmphen)$_2$-8HQ</td>
<td>17.3</td>
</tr>
<tr>
<td>GL019</td>
<td>Ru(dmphen)$_2$-8H-2MeQ</td>
<td>32.7</td>
</tr>
<tr>
<td>GL022</td>
<td>Ru(dmphen)$_2$bpy</td>
<td>5.4</td>
</tr>
<tr>
<td>GL023</td>
<td>Ru(dmphen)$_2$bp</td>
<td>15.2</td>
</tr>
<tr>
<td>GL039</td>
<td>Ru(dmphen)$_2$dpq</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*white light used
Active Species Determined by ESI-Mass Spectrometry

Mass spec experiments were carried out to determine the identity of the ejected ligand and active ruthenium species produced when the Ru(II) complexes were light activated. Strained octahedral complexes with accessible $^3$MC states (see Figure 2.2) were analyzed at micromolar concentrations to mimic conditions used for in vitro and in vivo experiments (see Chapters 4 and 5). The expected masses of the pure complexes were observed pre-exposure, and the light-activated ruthenium species and ejected ligand masses were observed post-exposure. As some ligands are observed better than others on the instrument, only qualitative observations were made about the percent abundance of the ejected ligands. For quantitative measurements of reaction kinetics, see Table 3.1.

Infusion of the model complex GL002 in the absence of light gives the expected masses of the complex ($M^+ = 743$ and $M^{2+} = 299$). Following white light activation, infusion of the same sample gives the expected, ejected ligand 6,6′-dimethyl-2,2′-bipyridine ($m/z = 185.0$ amu) as well as the activated Ru(bpy)$_2$ ($m/z = 414$) species capable of cross-linking DNA. The half-life of GL002 is 1.9 minutes (see Table 3.1) and complete conversion of reactant to product is qualitatively observed.

Complexes with the same Ru(bpy)$_2$ scaffold such as GL003, 006, 007, 010, and 014 exhibit similar ejection profiles, producing the more sterically hindered ligand and giving a single activated Ru(bpy)$_2$ species. Complexes such as GL003, 007, 010, and 014 that contain rigid, fused ring ligands eject more slowly (see Table
resulting in incomplete product conversion with two minutes of white light photo-activation.

Utilizing the more rigid bis-1,10-phenathroline (m/z = 462) backbone similarly yields the ejection of the single biquinoline ligand (m/z = 257) observed in complex GL011. Addition of methyl groups to this more rigid backbone such as the Ru(bis-2,9-dimethyl-1,10-phenanthroline)$_2$ backbone displayed in complexes GL018, 019, 022, 023, and 039, produce different active species. GL018 and 022 give a mixture of activated products when exposed to light. Complex GL018 ejects the 2,9-dimethyl-1,10-phenanthroline (m/z = 209) and 8-hydroxyquinoline (m/z = 146) ligands. Similarly, complex GL021 ejects 2,9-dimethyl-1,10-phenanthroline (m/z = 209) as well as 2,2'-bipyridine (m/z = 157). How this mixture of activated products affects the cellular potency is examined in Chapter 5.1. Complexes GL023 and 039 produce similar active species through the ejection of the dmphen ligand.

Complexes GL019 differs from GL018 only by the addition of a methyl group at the two-position on the 8-hydroxyquinoline ligand (see Table 2.1). While GL018 ejects a mixture of products, GL019 only ejects the quinoline ligand (m/z = 160). These complexes have similar in vitro IC$_{50}$ results (31 μM, see Table 4.1) forming cross-links to DNA. In addition to cross-links, GL019 also produces single strand breaks. Both complexes show potential as traditional chemotherapeutics (not light activated) and are potent on A549 cells, showing nanomolar IC$_{50}$'s (see Table 5.5). For a complete discussion on the activities of the quinoline complexes, see Chapter 5.1 A549 Growth Inhibition Assays.
The ejection properties of complexes with different overall charge states such as GL005 and 008 (overall charge = 2⁻ and 0 respectively) were also examined (see Table 2.2). Complex GL005 was found to eject the 6,6'-dimethyl-2,2'-bipyridine ligand \( m/z = 185 \), while complex 008 ejected a 2,2'-bipyridine ligand \( m/z = 157 \).

A summary of the complexes and the ligands ejected with a qualitative estimate of the percent conversion to the photo-activated complex with two minutes of white light are displayed in Table 3.2.

Table 3.2: Ejection products observed by ESI-MS

<table>
<thead>
<tr>
<th>Ruthenium Complex Code</th>
<th>Name</th>
<th>Ejected ligand</th>
<th>Estimated Percent Abundance of Ejected Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL002</td>
<td>Ru(bpy)_2dmbpy</td>
<td>dmbpy</td>
<td>100</td>
</tr>
<tr>
<td>GL003</td>
<td>Ru(bpy)_2dmdpq</td>
<td>dmdpq</td>
<td>60</td>
</tr>
<tr>
<td>GL005</td>
<td>Ru(bpds)_2dmbpy</td>
<td>dmbpy</td>
<td>100</td>
</tr>
<tr>
<td>GL006</td>
<td>Ru(bpy)_2-3,3’dmbpy</td>
<td>3,3’dmbpy</td>
<td>60</td>
</tr>
<tr>
<td>GL007</td>
<td>Ru(bpy)_2dmphen</td>
<td>dmphen</td>
<td>40</td>
</tr>
<tr>
<td>GL008</td>
<td>Ru(bpy)_2bcds</td>
<td>bcds</td>
<td>50</td>
</tr>
<tr>
<td>GL010</td>
<td>Ru(bpy)_2dmdppz</td>
<td>dmdppz</td>
<td>90</td>
</tr>
<tr>
<td>GL011</td>
<td>Ru(phen)_2biq</td>
<td>biq</td>
<td>50</td>
</tr>
<tr>
<td>GL013</td>
<td>Ru(bpy)_22,2’biq-3,3’dca</td>
<td>2,2’biq-3,3’dca</td>
<td>10</td>
</tr>
<tr>
<td>GL014</td>
<td>Ru(bpy)_2bc</td>
<td>bc</td>
<td>40</td>
</tr>
<tr>
<td>GL018</td>
<td>Ru(dmphen)_28HQ</td>
<td>8HQ/dmphen</td>
<td>60</td>
</tr>
<tr>
<td>GL019</td>
<td>Ru(dmphen)_2-2-Me-8HQ</td>
<td>2MeHQ</td>
<td>50</td>
</tr>
<tr>
<td>GL022</td>
<td>Ru(dmphen)_2bpy</td>
<td>bpy/dmphen</td>
<td>80</td>
</tr>
<tr>
<td>GL023</td>
<td>Ru(dmphen)_2bp</td>
<td>dmphen</td>
<td>50</td>
</tr>
<tr>
<td>GL039</td>
<td>Ru(dmphen)_2dpq</td>
<td>dmphen</td>
<td>80</td>
</tr>
</tbody>
</table>
3. Reaction of GL002 with Mixed Nucleosides by ESI-MS

Cisplatin is known to preferentially react with the N7 position of guanosine (see Figure 1.1). To a lesser extent, the complex also has an affinity for the N1 and N7 positions of adenosine as well as the N3 position of cytidine. Cisplatin has limited to no reactivity with thymidine. To determine which bases GL002 reacts with, a 2:1 ratio of GL002: individual nucleosides (guanosine, adenosine, cytidine, or thymidine) were studied via ESI-MS. Dark control experiments were ran parallel with light activated samples and incubated for one hour before MS analysis. All dark control samples displayed similar results. Masses of GL002 (M+ m/z = 597, M2+ m/z = 298) were observed in all samples with the individual nucleoside masses (adenosine m/z = 268, thymidine m/z = 242, cytidine m/z = 242, guanosine m/z = 284). Infusion of light activated reactions yielded complexes with all of the bases, though to a much lesser extent with thymidine. The masses observed for photo-activated GL002 are the active species, Ru(bpy)2 (m/z = 414) and the ejected ligand dmbpy (m/z = 185). Reactions with adenosine and photo-activated GL002 rendered a complex masses of m/z = 680 (expected m/z = 681), which corresponds to the Ru(bpy)2 (m/z = 414) active species plus adenosine (m/z = 268). The GL002-thymidine complex was found at very low abundance (m/z = 655, expected m/z = 656). Reactions with GL002-cytidine yielded a complex mass of m/z = 656 (expected 657). Guanosine produced the most abundant signal with M+1 m/z = 696 (expected 697). This was expected due to cisplatin’s preference to the base.
4. Reaction of GL002 with Mixed Nucleosides by LC-MS

To determine the nucleoside preference of GL002, the complex was activated and added to a mixture of the individual bases (A, C, T, G combined in equal parts), and analyzed on the LC-ESI mass spectrophotometer. 1200 μM GL002 was added to the nucleoside mixture (at 40 μM per base) following photoejection (1:1 complex: individual base) and injected. A mixed nucleoside-only sample was prepared at 40 μM per base and injected to serve as a standard and to calculate the percent recovery of the individual bases (see Figure 3.3). Retention times observed of the injected bases are: Cytidine, 13.8 minutes; Adenosine, 19 minutes; Guanosine, 19.8 minutes; Thymidine, 22.1 minutes (the slight shift in retention times of the bases observed upon the addition of GL002 in Figure 3.3b is due to inefficient equilibration of the column prior to injection. The method should be extended to 60 minutes, returning the gradient to 5% B at 55 minutes to allow adequate column equilibration prior to subsequent injections. Complex masses similar to those found above were observed with all nucleosides in low abundance. Addition of the peak at RT = 22.8 minutes contains the mass of the active species Ru(bpy)$_2$ plus guanosine (m/z = 696) is displayed in Figure 3.3c. Percent recovery was calculated using the Varian MS Workstation software. Preference to guanosine is observed through an 8% loss of guanosine in the base: complex sample when compared to the standard.
Figure 3.3: a) 40 μM mixed nucleosides (peaks in order: C, A, G, T); b) 1:1 GL002 with mixed nucleosides (C, A, G, 002 plus G, T; c) GL002: guanosine observed masses
5. Light Titration of GL002 monitored by ESI-MS

In order to determine the reactivity of GL002 with light by ESI-MS, a photoactivation experiment was performed while flowing sample into the spectrometer. This light titration experiment was carried out using the aforementioned ESI-MS setup with the addition of a flashlight to provide a light source to photo-activate the ruthenium complex. Dual ion monitoring mode was utilized to observe the dissociation of the complex. The m/z = 299 ion corresponds to the unactivated M²⁺ complex. The m/z = 185 ion corresponds to the ejected 6,6'-dimethyl-2,2'-bipyridine ligand. The isosbestic point observed at ~1.5 minutes parallels the half-life observed in Table 3.1. This mass screening analytical technique provides both the kinetics and the identity of the active species produced by the ruthenium complexes.

Figure 3.4: Light titration of GL002
6. Experimental

Absorbance measurements were obtained using an Agilent 8353 UV-Vis Spectrophotometer equipped with Agilent Chemstation Version B.02.01 sp1 software using a 1 cm cuvette. The instrument was blanked on the solvent used in the specific experiment. Compound concentrations were ca. 30 μM, and the initial dark control was scanned followed by exposure to blue light with Dell 1410X projector/200 W light source. Samples were placed 12 inches from the light source. Scans were taken periodically at set times to monitor the development of the degradation species and active species products until no change in the spectra was observed. Kinetics were calculated using Prism software to give photochemical half-lives.

Mass spectrometry experiments were carried out on a Varian 1200L Quadrupole MS/MS ESI mass spectrometer equipped with Varian MS Workstation Version 6.42 and Harvard Apparatus Pump 11 syringe pump. ~1 mg/mL complexes were prepared (chloride salts in water/PF₆ salts in acetonitrile) and kept from light. Micromolar solutions of each complex were infused in 80:20:0.1% methanol:water:formic acid before and after two minute light exposure with a 410 W light source (see Ch. 5 In vivo experimental) to observe the initial and light activated complex masses.

Reactions with nucleosides:

Guanosine (cas 118-00-3) was obtained from Alfa Aesar. Adenosine (cas 58-61-7) and cytidine (cas 65-46-3) were obtained from Sigma. Thymidine (cas 50-89-63)
5) was obtained from TCI. All reagents were used without further purification. Stock DMSO solutions of the individual nucleosides were prepared. Mixtures of the individual bases with GL002 were prepared at 1:2 (150 μM nucleoside: 300 μM GL002) ratios. Dark control samples were prepared through the addition of the nucleoside with GL002 and protected from light wrapped in aluminum foil. Light activated samples were prepared through the addition of the nucleoside with GL002 and placed under the aforementioned Dell projector for 1 hour. Samples were infused in 80:20:0.1% methanol:water:formic acid on the previously mentioned ESI mass spectrophotometer. Dark control samples were infused under low light room conditions with the syringe wrapped in aluminum foil.

Nucleosides used are described above. 1:1 GL002 (1200 μM): nucleoside (300 μM each) concentrations were used to obtain the results. Using stock DMSO solutions of the nucleosides, a mixture was prepared at 300 μM per base and was added to photo-ejected GL002 in dH2O and allowed to incubate at 37 °C for 2 hours. A nucleoside only sample was prepared at identical concentration of 300 μM per base in dH2O to serve as a standard and to calculate percent recovery. A blank solution was prepared to mimic DMSO concentrations in dH2O. The experiment was carried out with the previously mentioned ESI mass spectrophotometer with the addition of the two Varian ProStar pump (model 210). 10 μL were injected on a C18 column (Column Technologies Inc., 5μm 120 A, 4.5x25 cm, part number CTI0DS546250) at 0.25 mLmin⁻¹ using the following gradient:
Table 3.3: HPLC gradient used for GL002:mixed nucleoside HPLC-Ms experiment

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>0.1 % Formic Acid/miliQ-H$_2$O</th>
<th>0.1 % Formic Acid/MeCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>70</td>
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<tr>
<td>55</td>
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</tbody>
</table>

Light Titration of GL002:

Micromolar solutions of GL002 were infused in 80:20:0.1% MeOH:dH$_2$O:Formic Acid and acquired on the previously mentioned ESI mass spectrophotometer. Sample was infused in the dark and was exposed to light with a standard Rayovac IN2 flashlight. The photo-ejection of GL002 was observed following m/z ions 299 (M$_{2+}$) and the dissociated ligand dmbpy (m/z = 185). Curves were generated using Prism software using the ‘one phase association’ equation.
Chapter 4: *In Vitro* Studies of Light-Activated Ruthenium Complexes

Other scientists including Dr. David Heidary and Erin Wachter contributed to this chapter.

**Introduction**

The goal of this project is to synthesize photoactive complexes that form cross-links with DNA, leading to cell death. A distinction between dark and light activated DNA damage is required for successful photodynamic therapy. Dark toxicity should be minimized in order to avoid unnecessary damage to the healthy tissue surrounding the area to be treated. In order to test these effects, *in vitro* DNA damage studies were performed using the pUC19 plasmid DNA.

**4.1. DNA Damage Studies by Gel Electrophoresis**

*In vitro* gel electrophoresis experiments were carried out with pUC19 plasmid DNA to analyze the DNA damage caused by the synthesized ruthenium complexes either in the dark or through light activation. The activity of the complexes was compared to the chemotherapeutic cisplatin, the prototypical inorganic DNA damaging agent. Cisplatin cross-links DNA, which results in the induction of cell death, but a significant drawback is that it does not distinguish between healthy and cancerous cells.\(^1\) Unwinding of platinated pUC19 DNA is observed through the decreased mobility of the DNA in the agarose gel with increasing cisplatin concentrations (see Figure 4.1). The decreased mobility of the DNA is a result of increasing platination of the DNA with increasing concentrations.
of cisplatin. An IC₅₀ value of cisplatin was determined based on the concentration of the compound that resulted in the pUC19 plasmid existing in a state where it was unwound by 50%. This was observed at 31 μM with the complete unwinding occurring at 100 μM.

Figure 4.1: Cisplatin with pUC19 DNA

To determine the type of DNA interaction or damage that could occur with the ruthenium complexes, DNA damage was induced with other control compounds and agents. The metal complex copper phenanthroline (Cu(phen)₂) is known to produce single strand breaks by nicking the DNA forming the relaxed circular plasmid DNA.² This complex serves as a standard for single strand breaks to the pUC19 DNA and is included on all gels. Alternatively, complexes that form double strand breaks are highly desirable. This effect is included as a standard on gels through the use of the restriction enzyme EcoRI.³ Severe damage to the DNA through double strand breaks produces linear DNA leading to cell death. Double strand breaks require homologous recombination or nonhomologous end-joining repair mechanisms. This type of damage is most severe because neither strand can serve as a template for repair, resulting in cell death upon the subsequent cell cycle.⁴ Complexes that display this ability should be addressed in future work.
40 μg/mL pUC19 DNA was dosed with increasing amounts of each ruthenium complex followed by irradiation with blue light for one or three hours. Initial samples were pulled before light activation, and were protected from light to serve as the dark control. Due to the numerous compounds synthesized, this chapter will be divided into the following parts: 1) by active species a) (Ru(bpy)₂ and b) Ru(dmphen)₂, 2) complexes that intercalate, and 3) complexes that produce single strand DNA breaks. A comprehensive table is provided at the end of the chapter that summarizes the type of damage and IC₅₀’s observed at three hours (Table 4.1). The digital images of agarose gels for the dark control, one and three hour time points are included in Figure 4.6. This figure contains all gels produced by complexes in this thesis. These gels provide information on the types of DNA damage the complexes create and the concentration of the compound required to achieve the effect. Combining this mechanistic information with the cell viability can provide insight into the in vivo results shown in the A549 cell viability assays (see Chapter 5.1).

1a) Complexes that form the Ru(bpy)₂ backbone upon light activation

Complexes producing similar active species (for example, Ru(bpy)₂ see Table 3.2, ESI-MS Data) are highlighted in Figure 4.2. Complexes GL002, 003, 006, 007, and 014 produce the same Ru(bpy)₂ active species and display cross-linking ability with pUC19 DNA with IC₅₀ values of 15, 30, 30, 30, and 15 μM, respectively on the three hour gel.
Figure 4.2: Complexes with Ru(bpy)$_2$ active species

GL002

GL003

GL006

GL007

GL014
The one and three hour gels for GL002 are similar due to the kinetic ability of the complex to readily produce the active species ($t_{1/2} = 1.9$ minutes, see Table 3.1), which is capable of photo-binding DNA. GL003, 006, and 007 show similar effects, but with less potency to the pUC19 DNA with IC$_{50}$ values of 30 μM. Since the same species are generated, it is surprising that the IC$_{50}$ values are not equivalent. This disconnect is explained by the fact that GL003, 006, 007, and 014 are kinetically slower to produce the active species with $t_{1/2}$ values of 62, >232, 128, and 60 minutes respectively (see Table 3.1). This decreased efficiency to produce the active species results in the differences observed in the one and three hour time points associated with the complexes’ gels. The slow ejection is correlated with the more planar ligands used in the complex (see Table 2.2). The slight smearing of GL003 at the 3 hours time point is indicative of the intercalating ability of the dmdpq ligand into the base stack of the DNA and the subsequent unwinding of the DNA. Smearing due to intercalation is also observed in its’ non-photoejecting analogue, GL021. This complex is an efficient single strand DNA breaker and has an IC$_{50}$ of 30 μM. The gels for GL003 and 007 display both cross-linking and single strand breaks at three hours with the pUC19 DNA. It displays the anticipated single strand breaks associated with other ruthenium-phenanthroline complexes. This is attributed to the generation of reactive oxygen species (ROS, see section 3, complexes that produce single strand DNA breaks with pUC19 DNA). Complex GL014 also shows similar cross-linking to GL002 in addition to single strand breaks observed in the three hour gel. The ability to bind DNA and produce single strand breaks with the addition of the bathophen ligand might account for the increased dark A549 cell
viability that is observed (dark IC₅₀ value = 9.3 μM, see Table 5.5). Complexes containing the bis-bathophen backbones should be synthesized to increase potency. The issue with increased dark toxicity could possibly be relegated through the addition of negatively charged ligands.

1b) Complexes that produce the Ru(dmphen)₂ backbone

Other complexes containing similar backbones are highlighted in Figure 4.5. Complexes GL018, 019, 022, 023, and 039 contain the Ru(dmphen)₂ backbone. A ~50 nm red shift in the absorbance is gained through the addition of the dmphen backbone (see Figure 3.2).
Figure 4.3: Complexes containing the Ru(dmphen)_2 backbone

Complexes GL018 and 019 are quinoline-containing complexes that cross-link pUC19 DNA with IC_{50} values of 30 μM at three hours. GL019 produces single strand breaks in addition to cross-links. Quinoline ligands are known to have cytotoxic capabilities and are discussed in Chapter 2.1. Both complexes display in vitro IC_{50} values of 30 μM and considerable toxicity to A549 cells with IC_{50} values of
0.3 and 0.6 μM respectively (see Table 5.5). Complexes GL022 and 023 are equally efficient at cross-linking pUC19 DNA with IC_{50} values of 15 μM and are considerably potent on A549 cells with IC_{50} values of 1.2 μM and 0.2 μM (see Table 5.5) respectively. The DNA binding/intercalation observed in complex GL023 is thought to stem from the addition of the bp ligand (see Table 2.2). GL039 efficiently cross-links pUC19 DNA as well, with an IC_{50} value of 15 μM at three hours. In addition to cross-links, the complex also produces single strand breaks and intercalates DNA. The increased DNA affinity stems from the addition of the planar dpq ligand to the Ru(dmphen)_{2} backbone and produces similar intercalating results observed in similar complexes GL003, 009, and 010. The increased DNA affinity is observed in the A549 cell viability experiment with a dark IC_{50} result of 49.4 μM (see Table 5.5).

2) Complexes that intercalate pUC19 DNA

Complexes GL009 and 010 were synthesized with the purpose of generating compounds that strongly interact with the DNA duplex through intercalation. These compounds contain the dppz ligand (see Table 2.1), which is a large planar system that allows for intercalation through pi stacking with the bases of DNA. DNA binding affinity values of 10^{8} M^{-1} have been reported in the literature for similar dppz containing complexes. The effects of GL009 and 010 are shown in Figure 4.4.
Figure 4.4: pUC19 Intercalating ruthenium complexes

These compounds display similar results to GL003, where smearing of the DNA plasmid was observed in the gels. While GL009 shows no cross-linking because it is not photoactive, GL010 can slowly produce the same Ru(bpy)$_2$ active species ($t_{1/2} = > 6$ hrs), see Table 3.1) resulting in the observed, although slight cross-linking at the three hour time point. This is consistent with the narrow in vivo phototherapeutic window of 2.1 for GL010 (see Table 5.5).

3) Complexes that produce single strand DNA breaks with pUC19 DNA

Ruthenium complexes can generate singlet oxygen, producing single strand breaks in DNA. This type of damage from the evolution of singlet oxygen is known in the literature. Complexes GL004, 005, 011 and 021 display single strand breaks and are highlighted in Figure 4.5.
Figure 4.5: Ruthenium Complexes Producing Single Strand Breaks in pUC19 DNA

GL004, 011, and 021 have similar planar co-ligands (phen and dpq) as the Cu(phen)$_2$ standard and yield similar single strand breaks with IC$_{50}$ values of 15 and 30 μM respectively. Complex GL005 was synthesized to create an active species that was negatively charged to assess the importance of charge state in interacting with the DNA. The IC$_{50}$ value for complex GL005 is approximately 60 μM at the three hour time point (note that only about 10% of the pUC19 DNA is converted to single strand). Producing the active species of GL005 is kinetically, very efficient, with a
$t_{1/2}$ of 0.6 minutes (see Table 3.1). The complex contains the bis-bathophendisulfonate backbone to give an overall charge of $-2$, while a majority of the complexes discussed in this work have an overall charge of $+2$. The yield of ssDNA produced by GL005 is low compared to GL004. This is thought to be due to the photoejection, which decreases the yield of $^{1}O_2$. There is also a decreased electrostatic interaction with the DNA through the addition of the negatively charged backbone. This would suggest that GL005 would have very low potency in cell studies, but a 150-fold window is observed in the A549 cell viability assay (see Table 5.5). Limited dark toxicity (200 μM) is also observed in A549 cells supporting the decreased affinity through the negatively charged backbone. Additional complexes with similar backbones should be synthesized to prove this hypothesis.

Complex GL011 shows efficient cross-linking at an IC$_{50}$ value of 30 μM with single strand breaks occurring at higher concentrations. The Ru(phen)$_2$ backbone with the addition of a biquinoline ligand shifts the absorbance to the red by ~60 nm (see Figure 3.2). A phototherapeutic ratio of 5.4 (see Table 5.5) is observed in the A549 cell viability assay. Similar derivatives will be important in future research due to the deeper tissue penetrating nature associated with red shifted absorbances. The IC$_{50}$’s and type of damage observed at three hours is summarized in Table 4.1.
Table 4.1: Summary of IC\textsubscript{50} Values and Types of Damage Observed at Three Hours

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>IC\textsubscript{50} (\mu M)</th>
<th>Type of Damage Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>31</td>
<td>Cross-link</td>
</tr>
<tr>
<td>GL002</td>
<td>15</td>
<td>Cross-link/S.S Breaks</td>
</tr>
<tr>
<td>GL003</td>
<td>31</td>
<td>Cross-link/S.S Breaks/Intercalate</td>
</tr>
<tr>
<td>GL004</td>
<td>15</td>
<td>S.S. Breaks</td>
</tr>
<tr>
<td>GL005</td>
<td>31</td>
<td>S.S. Breaks</td>
</tr>
<tr>
<td>GL006</td>
<td>31</td>
<td>Cross-link/S.S Breaks</td>
</tr>
<tr>
<td>GL007</td>
<td>31</td>
<td>Cross-link/S.S Breaks</td>
</tr>
<tr>
<td>GL008</td>
<td>62</td>
<td>Cross-link/S.S. Breaks</td>
</tr>
<tr>
<td>GL009</td>
<td>&lt;7.5</td>
<td>Intercalate</td>
</tr>
<tr>
<td>GL010</td>
<td>&gt;62</td>
<td>Intercalate/Cross-link</td>
</tr>
<tr>
<td>GL011</td>
<td>31</td>
<td>Cross-link/S.S. Breaks</td>
</tr>
<tr>
<td>GL013</td>
<td>No Effect Observed</td>
<td>No Effect Observed</td>
</tr>
<tr>
<td>GL014</td>
<td>15</td>
<td>Cross-link/S.S Breaks</td>
</tr>
<tr>
<td>GL018</td>
<td>31</td>
<td>Cross-link</td>
</tr>
<tr>
<td>GL019</td>
<td>31</td>
<td>Cross-link/S.S Breaks</td>
</tr>
<tr>
<td>GL021</td>
<td>31</td>
<td>Intercalate/S.S. Breaks</td>
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<tr>
<td>GL023</td>
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<td>Cross-link/Intercalate/S.S. Breaks</td>
</tr>
<tr>
<td>GL039</td>
<td>15</td>
<td>Cross-link/Intercalate/S.S. Breaks</td>
</tr>
</tbody>
</table>
Figure 4.6: Ruthenium Complexes with pUC19 DNA

Cisplatin

GL002

GL003

GL004

GL005
Figure 4.6: Ruthenium Complexes with pUC19 DNA (cont.)

GL006

GL007

GL008

GL009

GL010

GL011
Figure 4.6: Ruthenium Complexes with pUC19 DNA (cont.)

GL013

GL014

GL018

GL019

GL021

GL022
Figure 4.6: Ruthenium Complexes with pUC19 DNA (cont.)

GL023

GL039
4.2. Reaction of GL002 with Calf Thymus DNA Monitored by UV-Vis Spectroscopy

To determine the binding kinetics for the reaction of the activated ruthenium complex with DNA, 20 μM GL002 was reacted with 100 μM (base pairs) calf thymus (CT) DNA in 10 mM NaH₂PO₄ buffer at 37 °C. Control dark reactions with GL002 and CT DNA yielded very little change in the spectra over 24 hours and are shown in Figure 4.7a. Figure 4.7b displays the reaction of photo-activated GL002 upon the addition of CT DNA, where scans were taken over a period of seven hours to provide the kinetic profile for the reaction. The CT DNA reacts rapidly, as observed by the spectral change observed in Figure 4.7b. A decrease in absorbance around 400 nm with an increase absorbance around 490 nm is observed. To determine the reaction rate, data was collected in triplicate and the change in absorbance was plotted against time to create a time course for the reaction. The data was fit to an equation for a ‘one phase association’ using Prism software and is shown in Figure 4.7c. The calculated half-life for the reaction is 52 minutes. The selectivity of GL002 to only bind the DNA after light-activation serves as a prodrug model for PDT use.
Figure 4.7: Kinetic profile of GL002 with 100 μM Calf Thymus DNA: a) Dark reaction, b) Light activated 002 with CT DNA, c) Light activated kinetics

4.3 Reaction of GL002 with Guanosine Monitored by UV-Vis spectroscopy

Although cisplatin is capable of binding guanine, cytosine, and adenine, the preferred base is guanine.10 Cisplatin binds at the N7 position of the base and is capable of forming interstrand and intrastrand cross-links.11 Intrastrand cross-links between the N7 atoms of adjacent guanine residues produce distortion of the DNA backbone.1,12,12a

Once the binding kinetics for the reaction of GL002 with Calf Thymus DNA were determined (see Figure 4.7) and preference to guanosine was found through nucleoside selectivity experiments using LC-MS (see Figure 3.3), the binding kinetics for GL002 with guanosine was determined using UV-Vis spectroscopy. This was assessed by reacting 20 μM GL002 with 1 mM Guanosine in 10 mM NaH2PO4 buffer at 37 ºC. To ensure that the interaction of guanosine was mediated by the activated GL002, control dark experiments were performed with unactivated GL002. The results from this control showed that GL002 does not react significantly with guanosine over several hours. When GL002 was photo-activated, it reacted quickly.
with guanosine as observed by the spectral change in Figure 4.8b. Data was collected for two hours following the addition of activated GL002 with guanosine and the kinetics for the reaction were measured and plotted using the difference in absorbance at 495 and 410 nm. The curve was fit with ‘one phase association’ with Prism software. The calculated half-life of the reaction is 12.7 minutes.

Figure 4.8: Kinetic Profile of GL002 with 1 mM Guanosine: a) Dark reaction, b) Light activated 002 with guanosine, c) Light activated kinetics

4.4. Reaction of GL002 with Glutathione Monitored by UV-Vis Spectroscopy

Glutathione (GSH) is a sulfur containing tripeptide that is highly reactive and found in most cells. Present in cells at concentrations ranging from 1-30 mM, GSH plays several roles including antioxidation and maintenance of the redox state. One of the most important role of GSH is detoxification of carcinogens, and elevated levels of GSH lead to chemotherapeutic drug resistance and aid in cell survival. GSH is problematic to cisplatin as the soft thiol groups have a high affinity for the soft platinum(II) metal. Pearson’s values of hardness (η) for platinum$^{2+}$ and sulfur are 8.0 and 4.14, respectively. The large difference ensures good covalent overlap
between the Pt$^{2+}$ and S atoms. Cisplatin/GSH kinetics are known in the literature and are relatively fast at a $t_{1/2} = \sim 53$ minutes.$^{13c}$ To determine the binding kinetics of GL002, 16 mM GSH was reacted with 20 $\mu$M GL002 in NaH$_2$PO$_4$ buffer. Addition of GSH to inactivated GL002 yielded no activity over 48 hours (Figure 4.9a). The ability to evade detoxification agents like GSH while inactive may allow for effective dosing of PDT patients with the prodrug GL002. Addition of GSH to photo-activated GL002 yielded a slow reaction, with a $t_{1/2} = 247.6$ minutes. The Pearson’s value of hardness for Ru$^{2+}$ is slightly lower than that of Pt$^{2+}$ at 5.86.$^{15}$ However, this is an estimate for the metal only and not for a coordination complex with aromatic ligands, which affect the electron density of the metal center. This experimental results show that sulfur has a higher affinity for the platinum in cisplatin over ruthenium in polypyridyl complexes. This is consistent with some results in the literature that shows some ruthenium complexes prefer nitrogen to sulfur.$^{16}$

Figure 4.9: Kinetic Profile of GL002 with GSH: a) Dark reaction, b) Light activated 002 with GSH, c) Light activated kinetics
Chapter 4: In Vitro Experimental

In Vitro: Agarose Gel Electrophoresis Experimental

Materials:

The pUC19 plasmid DNA was obtained from ATCC and was purified with the Maxi Prep kit by Qiagen. Buffered solutions containing, tris base, and acetic acid were obtained from Sigma, VWR or Fisher and were prepared to the appropriate concentrations using Glazer Lab Agarose Gel Electrophoresis Protocols. Ingredients for the DNA loading dye include bromophenol blue, xylene cyanol, and glyceron were obtained from VWR and prepared to appropriate concentrations using Glazer Laboratory Protocols. Single strand breaks of the plasmid (relaxed circle) were observed using copper phenanthroline (Cu(phen)$_2$) obtained from Sigma and prepared via the reaction outlined below. Reagent grade dithiothreitol (DTT) and H$_2$O$_2$ were obtained from VWR. Double strand (linear DNA) breaks to the pUC19 plasma DNA were observed using the endonuclease enzyme EcoR1 obtained from Fisher and was prepared per protocol outlined below. A DNA ladder from Promega was used for mass estimation of DNA fragments produced and was prepared through the procedure outlined below. Pure agarose was obtained from Fisher and prepared using the protocol outlined below. Gel rins used for running the gels were also obtained from BioRad. Gels were stained with ethidium bromide (Fisher) and digitally imaged.
Single Strand DNA Break Cu(OP)$_2$ Reaction:

Single strand breaks of the pUC19 plasmid DNA were created through the incubation of 40 μg/mL of pUC19 in 10 mM phosphate buffer, pH 7.4 mixed with 8 μM Cu(phen)$_2$. The reaction was initiated with the addition of 4 μL of 5 mM DTT and 4 μL of 5 mM H$_2$O$_2$. The reaction mixture was vortexed for 10 seconds and allowed to incubate at room temperature for 30 minutes. Prior to loading onto the agarose gel, 6 μL of DNA loading dye was added to the sample.

Double Strand DNA break EcoR1 Reaction:

Double strand breaks of the pUC19 plasmid DNA were accomplished through the incubation of 40 μg/mL of pUC19 with 8 μL of EcoR1, a DNA restriction enzyme, mixed with 10 μL 10X EcoR1 buffer. The appropriate amount of dH$_2$O was added to bring the final volume to 100 μL. The solutions were thoroughly mixed and allowed to react at 37 °C for 90 minutes on a heat block. Upon completion and prior to use, 20 μL of 6X DNA loading dye was added.

DNA Ladder for Mass Estimation of DNA Fragments:

Mass estimation of the DNA fragments produced was observed through the use of a 1Kbase pair ladder. 10 μL of the 1Kbase pair ladder was diluted 10X with 20 μL of 6X loading dye and dH$_2$O.
Agarose Gel Preparation:

1% agarose gels were prepared by mixing 0.5 g agarose with 50 mL of 1X tris acetate buffer and heated to boiling to ensure complete dissolution in a microwave. The solution was then poured into the gel deck with a 15 well comb in place and allowed to solidify for at least 30 minutes. Once solidified, the gel was placed in the gel rig filled with 1X tris acetate running buffer.

Sample Preparation: DNA dosing and Light Activation

A dilution series was prepared in clear, 96-well flat bottom plates (clear, Costar) of the following ruthenium complex concentrations in 10 mM Na₂PO₄ buffer: 500 μM, 250 μM, 125 μM, 62.5 μM, 31.3 μM, 15.6 μM, 7.8 μM, and 0 μM. pUC19 plasmid DNA concentration was added to each well at 40 μg/mL (final volume 90 μL). A 30 μL aliquot was removed for each dose point to serve as the dark control. The remaining 60 μL were exposed to blue light from a 200 W Dell projector. 30 μL sample aliquots were removed at 1 hour and 3 hours of light activation. After light exposure the samples were allowed to react at room temperature overnight. Prior to loading on the agarose gel 6 μL of 6X DNA loading dye were added to each 30 μL sample aliquots with 8 μL of the samples being loaded into the gel well. Typical order of the loaded samples is: DNA ladder, EcoR1, Cu(OP)₂, 0 μM ruthenium complex to 500 μM ruthenium complex, DNA ladder. The gels were run for 75 minutes at 100 mV. Gels were stained with 7.5 μL of ethidium bromide in 150 mL 1X Tris acetate Buffer for 40 minutes followed by de-staining of the gels with a fresh 150 mL aliquot of 1X tris acetate buffer for 30 minutes and imaged digitally.
4.2 In Vitro: GL002 with Calf Thymus DNA by UV-Vis spectroscopy: Experimental

Reaction kinetics were obtained for GL002 with Calf Thymus DNA in NaH$_2$PO$_4$ buffer at pH 7.4 at 37 °C using an Agilent 8353 UV-Vis spectrophotometer equipped with Chemstation B.02.01 software and a temperature controller peltier (Agilent 89090A). Calf thymus DNA was obtained from ATCC and sonicated for 30 minutes to produce uniform breaks in the DNA. The instrument was blanked on 7 μL of 1M NaH$_2$PO$_4$ at pH 7.4 (10 mM) and 691 μL of deionized water in a small volume cuvette. 2.1 μL of GL002 (6.55 mM) was added to the blank solution to give 002: NaH$_2$PO$_4$ concentrations of 20 μM: 10 mM respectively. Following an initial scan, the cuvette was placed under the Dell projector (see Chapter 3.1) and exposed to blue light until no change was observed in the UV spectra confirming the complete conversion of the complex to the Ru(bpy)$_2$ active species. The reaction was initiated through the addition of 5.8 μL sonicated calf thymus DNA (5160 μM in base pairs) to 294.2 μL of the compound/buffer solution above to give the final 002:CT DNA: NaH$_2$PO$_4$ concentrations of 19.6 μM: 100 μM: 9.8 mM respectively. The cuvette was placed in the sample holder with the peltier temperature controller set to 37 °C. Scans were taken periodically until no spectral change was observed. Triplicate measurements were obtained and curves were fit using one phase association in Prism software.

4.3 In vitro; GL002 with Guanosine by Uv-Vis spectroscopy: Experimental

The reaction kinetics were obtained for GL002 with guanosine (Alfa Aesar, CAS 118-00-3) in NaH$_2$PO$_4$ (Sigma) buffer at pH 7.4 at 37 °C using the previously
mentioned UV-Vis Spectrophotometer. The instrument was blanked on 3 µL of 1 M NaH₂PO₄ (10 mM) and 296.1 µL of deionized water in a small volume cuvette. 0.92 µL of 6.55 mM GL002 (20 µM) was added to the cuvette and an initial scan was taken. The sample was place under the Dell projector (see Chapter 3.1) under blue light until no spectral change was observed and photoejection was complete. 9 µL of a 33.5 mM guanosine stock in DMSO was added to 291 µL of the GL002/NaH₂PO₄ buffered solution to give GL002: guanosine: NaH₂PO₄ concentrations of 19.4 µM: 1 mM: 9.7 µM respectively. The cuvette was placed in the sample holder with the peltier set to 37 °C. Scans were taken periodically until no spectral change was observed. Triplicate measurements were obtained and the resulting curves were fit using one phase association in Prism software.

4.4 In Vitro: GL002 with Glutathione

The reaction kinetics were obtained for GL002 and L-glutathione reduced (GSH, Sigma, CAS 70-18-8) in NaH₂PO₄ (Sigma) buffer at pH 7.4 at 37 °C using the previously mentioned UV-Vis spectrophotometer. The instrument was blanked on 3 µL of 1 M NaH₂PO₄ (10 mM) and 2498.13 µL of deionized water in a 3 mL cuvette. 0.92 µL of 65.5 mM GL002 (20 µM) was added to the cuvette and an initial scan was taken. The sample was place under the Dell projector (see Chapter 3.1) under blue light until no spectral change was observed and photoejection was complete. 480 µL of 100 mM GSH (16 mM) was added to the mixture and the cuvette was place in the sample holder with the peltier set to 37 °C. Scans were taken periodically until no
spectral change was observed. Triplicate measurements were obtained and the resulting curves were fit using the one phase association in Prism software.

Chapter 5: In Vivo Studies of Light-Activated Ruthenium Complexes

Dr. David Heidary contributed to this chapter.

Introduction

In vitro results indicate that the synthesized ruthenium complexes are capable of cross-linking DNA when photo-activated. In order to test their in vivo activity, experiments were carried out on the A549 cell line. A549 or adenocarcinomic human alveolar basal epithelial cells are a non-small cell lung cancer cell line. These adherent cells are responsible for the diffusion of water and electrolytes across the alveoli of lungs and are cultured as a monolayer. The immortalized cell line was derived from an explanted tumor of a 58-year-old Caucasian male.17 The lung cancer cell line was chosen as a good model for photodynamic therapy as the lung is easily accessible with a light source and photodynamic therapy has been applied with success to lung cancer.18

Chapter 5.1: A549 Cytotoxicity Assays

Successful photodynamic therapy hinges on the ability to provide cytotoxic results only when the complexes are activated with light. As described in Chapter 2.2, multiple complexes have been synthesized to examine cell cytotoxicity in search of a good balance of light and dark toxicity. Due to the numerous compounds synthesized, this chapter will be divided and discussed as follows: 1) by active
species a) (Ru(bpy)$_2$ and b) Ru(dmphen)$_2$ and how the *in vivo* A549 assay results relate to structural design (see Chapter 2 Design) and photo-ejection kinetics (see Chapters 3); 2) *in vitro* pUC19 DNA results (see Chapter 4.1), with regards to complexes that crosslink DNA or produce single strand DNA breaks, 3) complexes with different overall charges, and 4) summary and future work. Complexes that fit the criteria for each feature will be grouped and discussed. Figures that correspond to relevant compounds discussed will be included in the text and all cytotoxicity results will be summarized in a comprehensive table included at the end of the Chapter. For all experiments, cisplatin was used as a non-light activated control; it displays a cytotoxic ratio of one as it displays no noticeable difference in the presence of light.

Figure 5.1: Cytoxicity curve of cisplatin

To test if only light activated complexes induced cytotoxicity, dark control experiments were carried out to observe efficacy between the light activated and unactivated complexes. Cell viability was determined through an ATP luciferase assay that produces luminescence in the presence of ATP. Detergent lysis was used to rupture the membrane gaining access to the cellular contents, and the enzyme luciferase was used to produce luminescence in the presence of ATP. Populations of
viable and dead cells are quantified through measuring luminescence with the Tecan plate reader.

1a: Complexes that produce the Ru(bpy)$_2$ backbone

Complexes GL002, 003, 006, 007, 008, 010 have similar backbones and form the same Ru(bpy)$_2$ active species following activation (Table 5.1).

Table 5.1: Complexes that produce the Ru(bpy)$_2$ active species upon light activation

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>Name</th>
<th>A549 Light (μM)</th>
<th>A549 Dark (μM)</th>
<th>A549 phototherapeutic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL002</td>
<td>Ru(bpy)$_2$dmbpy</td>
<td>0.6</td>
<td>250</td>
<td>417</td>
</tr>
<tr>
<td>GL003</td>
<td>Ru(bpy)$_2$dmdpq</td>
<td>1.2</td>
<td>250</td>
<td>216</td>
</tr>
<tr>
<td>GL006</td>
<td>Ru(bpy)$_2$-3,3'-dmbpy</td>
<td>30</td>
<td>250</td>
<td>8</td>
</tr>
<tr>
<td>GL007</td>
<td>Ru(bpy)$_2$dmphen</td>
<td>0.1</td>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td>GL008</td>
<td>Ru(bpy)$_2$bcds</td>
<td>56.3</td>
<td>64.5</td>
<td>1.2</td>
</tr>
<tr>
<td>GL010</td>
<td>Ru(bpy)$_2$dmdppz</td>
<td>22</td>
<td>47</td>
<td>2.1</td>
</tr>
<tr>
<td>GL014</td>
<td>Ru(bpy)$_2$bc</td>
<td>2.4</td>
<td>9.3</td>
<td>3.9</td>
</tr>
</tbody>
</table>
It is expected that complexes with similar backbones producing the same active species should have similar light IC₅₀ values. However, with these complexes sharing the similar backbone, correlations between the structure and ejection profiles and the \textit{in vitro} results can loosely be drawn to explain the potency observed in the \textit{in vivo} A549 assay. The general trend is that complexes that are slower to produce the active species are less effective at cross-linking DNA and are less potent compared to their faster ejecting derivatives. The model complex GL002 shows the largest phototherapeutic ratio for this group of complexes of 417. Light activation of the compound resulted in an IC₅₀ of 0.6 μM while the non-activated compound induced cytotoxicity with an IC₅₀ of 250 μM. A similar ratio is observed with GL003 (216), which has an IC₅₀ of 1.2 μM and dark unactivated IC₅₀ of 250 μM. The slight decrease in light toxicity of GL003 is attributed to the slower ejection profile of 60.9 minutes (see Table 3.1). This compliments the two-fold increases in the complex’s IC₅₀ \textit{in vitro} result (see Table 4.1). Complex GL007 is approximately 50 times slower to eject than GL002 (see Table 3.1), but is very potent against the A549 cell line once photo-activated (0.1 μM). The ejected dmphen ligand (see Table 3.2) appears to have an effect on the cell line, increasing the dark toxicity similarly.
to what is observed in the Ru(dmphen)$_2$ activated species (see Table 5.2). This may be attributed to the complexes’ dual mechanism of cross-linking and producing single strand breaks as seen in the in vitro results (Table 4.1). Complexes GL008 and 014 are structurally similar and differ only by overall charge through the addition of the sulfonate groups to GL008 (see Table 2.2). The negatively charged bcds ligand decreases the overall toxicity toward the cell line. This could be related to the electrostatic interaction with DNA, raising both the light and dark toxicity to 56.3 and 64.5 μM respectively. This compliments the in vitro results as well (IC$_{50}$ = 60 μM, see Table 4.1), possibly indicating that the negatively charged ligand has a decreased affinity towards DNA. The addition of the bc ligand in complex GL014 (see Table 2.2) has the opposite effect, decreasing both the light and dark toxicity. This complex is slow to eject at 59.7 minutes (see Table 3.1), but fairly potent when light activated (2.4 μM), and has considerably more dark toxicity resulting in a diminished phototherapeutic window of 3.9. GL023 incorporates a similar ligand and has considerable dark toxicity (see Table 5.2). Diminished potency compared to GL002 after light activation was observed for complexes GL006 and GL010, with IC$_{50}$ values of 30 and 22 μM respectively, despite the fact that they produce the same active species. This is attributed to their respective slow ejection profiles (t$_{1/2}$ values of 230 and >6 hrs respectively; see Table 3.1). On average these complexes are approximately 150 times slower than GL002 at producing the active species capable of cross-linking DNA. As shown in the in vitro results, GL006 has an IC$_{50}$ of 30 μM at 3 hours under blue light; two fold less effective that GL002 at three hours. The in vitro result of GL010 is > 60 μM at 3 hours, a difference of four when compared to
GL002 (see Table 4.1). It is thought that the dark toxicity stems from the extended, planar dmdppz ligands intercalating nature to bind DNA. This observation is evident in the three hour in vitro result (see Figure 4.1), as indicated by smearing of the DNA. For complexes GL006 and 010, irradiation times appear to be very important. To address the issue of complete conversion to the activated species, optimized irradiation times should be addressed in future cell assays of slow ejectors to ensure complete photo-activation of the complexes.

1b: Complexes that produce the Ru(dmphen)$_2$ backbone

A different approach was evaluated with complexes containing the Ru(dmphen)$_2$ backbone. Instead of the addition of one sterically hindered ligand added to the Ru(bpy)$_2$ backbone, steric clash was introduced through the addition of two sterically hindered ligands in the Ru(dmphen)$_2$ backbone, and then coupled with various unstrained ligands. Table 5.2 summarizes the complexes that produce the Ru(dmphen)$_2$ backbone upon light activation.
Table 5.2: Complexes that produce the Ru(dmphen)_2 active species upon light activation

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>Name</th>
<th>A549 Light (μM)</th>
<th>A549 Dark (μM)</th>
<th>A549 phototherapeutic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL018</td>
<td>Ru(dmphen)_28HQ</td>
<td>0.3</td>
<td>0.3</td>
<td>1</td>
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<tr>
<td>GL019</td>
<td>Ru(dmphen)_22-Me-8HQ</td>
<td>0.6</td>
<td>1.2</td>
<td>2</td>
</tr>
<tr>
<td>GL022</td>
<td>Ru(dmphen)_2bpy</td>
<td>1.2</td>
<td>24.5</td>
<td>20.4</td>
</tr>
<tr>
<td>GL023</td>
<td>Ru(dmphen)_2bp</td>
<td>0.2</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>GL039</td>
<td>Ru(dmphen)_2dpq</td>
<td>0.8</td>
<td>49.4</td>
<td>61.8</td>
</tr>
</tbody>
</table>

Figure 5.3: Cytotoxicity curves of example complexes containing the Ru(dmphen)_2 backbone

The Ru(dmphen)_2 backbone provided complexes that can be light activated, but dark toxicity was sacrificed. These complexes have narrow phototherapeutic windows and behave somewhat like traditional therapeutic metal complexes. The
quinoline ligands alone are known cytotoxic agents and are discussed in Chapter 2.1.\textsuperscript{19} GL018 and 019 were synthesized to evaluate the phototherapeutic ratio of the addition of these cytotoxic ligands with the strained backbone. On average, the ejection profiles of these complexes are 13 times slower than the model complex GL002 (see Table 3.1) and produce different photo-ejection products (see Table 3.2). GL018 produces a mixture of products upon light activation including the dmphen ligand (similar to complex GL007, see Table 3.2). This complex is potent in the A549 cell line with an IC\textsubscript{50} = 0.3 μM under both dark and light conditions, behaving like a traditional chemotherapeutic with no phototherapeutic window. The photo-ejection products of GL019 are different from 018 (see Table 3.2), ejecting only the 2-Me-8-HQ ligand, and providing a narrow phototherapeutic window of 2. This complex is twice as slow as GL018 at producing the active Ru(dmphen)\textsubscript{2} species (see Table 3.1) but maintains potency with a light activated IC\textsubscript{50} of 0.6 μM. Both of these complexes display similar affinity to pUC19 DNA with IC\textsubscript{50}’s of 30 μM observed in the \textit{in vitro} experiments (Table 4.1). Derivatives containing both of these ligands should be synthesized with negatively charged backbones such as Ru(bpds)\textsubscript{2} (see Table 2.1) to attempt to reduce the dark toxicity issues associated with these complexes. The complex GL022 ejects in a timely manner (5.4 minutes, see Table 3.1) and ejects a mixture of products (see Table 3.2). With this combination of ligands, dark toxicity is diminished, resulting in a phototherapeutic ratio of 20.4. Complex GL023 is slightly slower to eject at 15.2 minutes (see Table 3.1) and has a phototherapeutic window of 2.5. The addition of the bp ligand (see Table 2.1) is thought to contribute to the dark toxicity through increased DNA affinity as seen smearing of the DNA in
the in vitro gels (see Figure 4.6). A control complex of Ru(bpy)$_2$bp should be synthesized to observe the dark toxicity associated with the ligand. GL039 has a wide phototherapeutic window of 61.8 and is quick to eject at 3.2 minutes (see Table 3.1) producing one active species (Ru(dmphen)dpq, see Table 3.2). This complex contains a planar, intercalating ligand that has a high affinity to DNA as seen in the in vitro pUC19 results at three hours (IC$_{50}$ = 15 μM, see Table 4.1). A light activated in vivo IC$_{50}$ value of 0.8 μM is attributed to the ejection of the single dmphen ligand. The complex Ru(dpq)$_2$dmphen should be synthesized to observe how the intercalating dpq ligand starting material coupled with the dmphen ligand interact with DNA and cells.

2) Complexes that produce single strand DNA breaks

Several research groups are synthesizing complexes that produce single strand breaks through the generation of reactive oxygen species (ROS). These complexes are described in the literature.$^5$ Three complexes highlighted in this work, GL004, 005, and 021, also produce in vitro single strand DNA breaks (see Figure 4.5).
Table 5.3: Complexes that produce *in vitro* single strand DNA breaks

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>Name</th>
<th>A549 Light (μM)</th>
<th>A549 Dark (μM)</th>
<th>A549 phototherapeutic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL004</td>
<td>Ru(bpy)$_2$phen</td>
<td>40</td>
<td>250</td>
<td>6.3</td>
</tr>
<tr>
<td>GL005</td>
<td>Ru(bpds)$_2$dmbpy</td>
<td>1.3</td>
<td>200</td>
<td>154</td>
</tr>
<tr>
<td>GL021</td>
<td>Ru(bpy)$_2$dpq</td>
<td>123</td>
<td>123</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 5.4: Cytotoxicity curves of example complexes that produce *in vitro* single strand breaks

Of these complexes, GL004 is most efficient at producing the *in vitro* DNA breaks followed by GL021, and lastly GL005 (15, 30, and 60 μM respectively, see Table 4.1). However, there is a disconnect in this design approach based on the efficiency to produce *in vitro* single strand breaks and A549 cell cytotoxicity. The worst single strand breaker, GL005, is shown in Table 5.3 to produce the best phototherapeutic window of 154 (see cytotoxicity curve in Figure 5.5), followed by GL004 (6.3) and GL021 (1). GL005 is the only photoactive complex in the group
with a $t_{1/2}$ of 0.6 minutes (see Table 3.1). This complex contains the Ru(bpds)$_2$ backbone and carries an overall charge of -2 (see Table 2.2), ejecting the dmbpy ligand upon light activation (see Table 3.2). GL004 contains the phen ligand (see Table 2.1). This ligand is known to produce ROS, and a similar metal complex, Cu(phen)$_2$, is used as a standard to produce single strand breaks in the *in vitro* gel experiments (see Chapter 4 Experimental). GL021 has no distinguishable phototherapeutic window and contains an intercalating dpq ligand (see Table 2.1). The planar nature of the dpq ligand enhances DNA affinity leading to dark toxicity. This complex intercalates as evident in the smearing of the DNA in the gel and shows a fairly efficient *in vitro* IC$_{50}$ of 30 μM (see Table 4.1) for single strand DNA breaks. Based on this study, designing complexes based on their ability to produce single strand breaks does not correlate to *in vivo* cell cytotoxicity, at least in the A549 cell line. Additional cell lines should be screened with these complexes to confirm this analysis.

3) Complexes with different overall charges

The majority of the ruthenium complexes synthesized for this work have the overall oxidation state of +2. Complexes with overall neutral or negative charges are of interest due to their low electrostatic attraction with the negatively charged DNA backbone. Decreased DNA affinity should decrease dark toxicity prior to light activation. Negatively charged and zero charged complexes such as GL005 and 008 display this property with decreased dark toxicity IC$_{50}$ values seen below.
Table 5.4: Complexes with different overall charges to reduce DNA affinity

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>Name</th>
<th>A549 Light (μM)</th>
<th>A549 Dark (μM)</th>
<th>A549 phototherapeutic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL005</td>
<td>Ru(bpds)₂dmphen</td>
<td>1.3</td>
<td>250</td>
<td>154</td>
</tr>
<tr>
<td>GL008</td>
<td>Ru(bpy)₂bcds</td>
<td>56.3</td>
<td>64.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Figure 5.5: Cytotoxicity curves of complexes that contain different overall charge states.

The decreased dark toxicity of GL005 (overall charge of -2) could stem from the decreased ionic affinity to the DNA. This complex is potent when light activated (1.3 μM) and has minimal dark toxicity providing a phototherapeutic window of 154. A full series of complexes containing this backbone with the dmphen, bp, and bcds ligands (see Table 2.1) should be synthesized and screened on the A549 cell line to grasp an understanding of how the overall negative charge affects *in vivo* results. Complex GL008 has a net 0 charge, a t₁/₂ of 12.6 minutes (see Table 3.1), and ejects the bcds ligand upon light activation (see Table 3.2), producing the Ru(bpy)₂ active species. The negatively charged bcds ligand ejected appears to inhibit *in vivo*
toxicity seen in the model complex GL002 and other complexes producing the same active species (see 5.1a). To see how net 0 charged complexes affect pUC19 DNA and the A549 cell line, derivatives such as Ru(dmphen)$_2$bpds should be synthesized.

4) Summary and future work

The flexible coordination chemistry (see Chapter 2.1) of ruthenium offers a unique approach for photodynamic therapy in that DNA affinity can possibly be reduced or gained based on electrostatics as well as structural features of the complex. To obtain DNA affinity, modifications to the structure should include intercalating ligands. Increased dark toxicity can also be obtained through the addition of bathophen or quinoline ligands and decreased dark toxicity can be achieved through addition of negatively charged ligands. These functionalities should be examined in future works. Complexes displaying a red shifted absorption profile could improve the current PDT capabilities by penetrating deeper lying tumors.$^{20}$ Derivatives of complexes containing ligands that display cytotoxic activity should be coupled with essentially inert backbones. The rich nature of ruthenium polypyridyl chemistry is well known.$^{21}$ As such, libraries of complexes can be synthesized and tested for cell cytotoxicity. Combinatorial approaches are known and can be used to produce and screen large libraries of complexes.$^6$ A comprehensive table of IC$_{50}$ results can be found in Table 5.5.
Table 5.5: *In vivo* IC$_{50}$ Values

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>Name</th>
<th>A549 light (μM)</th>
<th>A549 dark (μM)</th>
<th>A549 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL001</td>
<td>Cisplatin</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>GL002</td>
<td>Ru(bpy)$_2$dm bpy</td>
<td>0.6</td>
<td>250</td>
<td>417</td>
</tr>
<tr>
<td>GL003</td>
<td>Ru(bpy)$_2$dmdpq</td>
<td>1.2</td>
<td>250</td>
<td>216</td>
</tr>
<tr>
<td>GL004</td>
<td>Ru(bpy)$_2$phen</td>
<td>40</td>
<td>250</td>
<td>6.3</td>
</tr>
<tr>
<td>GL005</td>
<td>Ru(bathophendisulfonate)$_2$dm bpy</td>
<td>1.3</td>
<td>250</td>
<td>192</td>
</tr>
<tr>
<td>GL006</td>
<td>Ru(bpy)$_2$-3,3'dm bpy</td>
<td>30</td>
<td>250</td>
<td>8</td>
</tr>
<tr>
<td>GL007</td>
<td>Ru(bpy)$_2$dm phen</td>
<td>0.1</td>
<td>8</td>
<td>80</td>
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<tr>
<td>GL008</td>
<td>Ru(bpy)$_2$bathocuprinedisulfonate</td>
<td>56.3</td>
<td>64.5</td>
<td>1.2</td>
</tr>
<tr>
<td>GL009</td>
<td>Ru(bpy)$_2$dppz</td>
<td>22</td>
<td>22</td>
<td>1</td>
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<tr>
<td>GL010</td>
<td>Ru(bpy)$_2$dmdppz</td>
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<td>47</td>
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<td>GL011</td>
<td>Ru(phen)$_2$biquinoline</td>
<td>4.2</td>
<td>22.5</td>
<td>5.4</td>
</tr>
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</table>
Table 5.5: *In vivo* IC$_{50}$ Values (cont.)

<table>
<thead>
<tr>
<th>Complex Code</th>
<th>Name</th>
<th>(a_{549}) light (μM)</th>
<th>(a_{549}) dark (μM)</th>
<th>(a_{549}) ratio</th>
</tr>
</thead>
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<tr>
<td>GL013</td>
<td>Ru(bpy)$_2$2,2’biq-3,3’dca</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>1</td>
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<tr>
<td>GL014</td>
<td>Ru(bpy)$_2$bathocuprione</td>
<td>2.4</td>
<td>9.3</td>
<td>3.9</td>
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<tr>
<td>GL018</td>
<td>Ru(dmphen)$_2$8HQ</td>
<td>0.3</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>GL019</td>
<td>Ru(dmphen)$_2$2Me8HQ</td>
<td>0.6</td>
<td>1.2</td>
<td>2</td>
</tr>
<tr>
<td>GL021</td>
<td>Ru(bpy)$_2$dpq</td>
<td>123</td>
<td>123</td>
<td>1</td>
</tr>
<tr>
<td>GL022</td>
<td>Ru(dmphen)$_2$bpy</td>
<td>1.2</td>
<td>24.5</td>
<td>20.4</td>
</tr>
<tr>
<td>GL023</td>
<td>Ru(dmphen)$_2$bathophen</td>
<td>0.2</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>GL039</td>
<td>Ru(dmphen)$_2$dpq</td>
<td>0.8</td>
<td>49.4</td>
<td>61.8</td>
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</tbody>
</table>

Chapter 5.2: *In Vivo* A549-GSH Cell Viability Assay

The A549 lung cancer cell line chosen for this work is known for elevated glutathione (GSH) levels. A seven-fold increase was found in the A549 cell line when compared to normal human lung fibroblast cell line (CCL-210). $^{14}$ Normal cellular levels of GSH range from 1-30 mM$^{13c}$ The many roles GSH plays in cellular activity are discussed in Chapter 4.4. Detoxification of antineoplastic agents such as cisplatin is performed by glutathione S-transferases that bind the metal and are removed
from the cell via the ATP dependent GS-X pump. Elevated levels of GSH hinder cytotoxicity of various antineoplastic drugs like melphalan, nitrogen mustard, and cisplatin. In addition to decreased cytotoxicity from these common chemotherapeutics, radiation therapy is also compromised as a result of elevated GSH levels.

To ensure the synthesized ruthenium complexes are not deactivated by GSH and to compare the in vivo effect of GSH on cisplatin and GL002, a dose response of GSH was performed where the amount of GSH was increased from 0-16 mM followed by the addition of the cytotoxic complexes. Cisplatin and GL002 concentrations were held constant at 20 μM. The IC50 of cisplatin in the absence of GSH was found to be 1.5 μM on A549 cells (see Table 5.5). Cisplatin results are shown in Figure 5.6a and are consistent with the literature in that cell viability increases with increasing amounts of GSH. Thus, cisplatin becomes a less efficient cytotoxic complex, with a GSH IC50 value of 4.3 μM. ~100% of cells are viable at the top GSH concentration of 16 mM.

GL002 was found to react minimally with GSH in dark experiments and slowly with GSH upon photo-activation in vitro, with a t1/2 = 247.6 minutes (see Figure 4.9b). The A549 IC50 value for the activated complex GL002 is 0.6 μM (Table 5.5). Figure 5.6b shows the GSH titration with photoactivated GL002. Glutathione seems to enhance cytotoxicity with light activated GL002. No effect was observed with unactivated GL002. This combination of in vitro and in vivo studies indicate that the complexes will not be deactivated by GSH both prior to and following light
activation. In contrast, addition of GSH was found to decrease the binding efficiency and cytotoxicity of cisplatin. The ability of the synthesized ruthenium complexes to evade detoxification by GSH while unactivated renders light activation of the complexes a viable PDT method. Similar in vitro pUC19 gel experiments were carried out by contributing scientists in our lab with similar results. Future in vivo A549 light activated studies should be performed to determine the amount of GSH required for the complete deactivation of photo-activated GL002. These results indicate that the ruthenium PDT strategy does not suffer the same inactivation by biological thiols as platinum agents at physiological concentrations of GSH. Future studies should focus on the ability of ruthenium agents to avoid alternative detoxification mechanisms associated with cisplatin resistance.

Figure 5.6: GSH Titration with a) CP and b) GL002
Chapter 5 Experimental

A549 Growth Inhibition Assays Experimental

A549 cell viability assay

A549 cells were obtained from Dr. Rolf Craven and were grown in Dulbecco Minimum Essential Medium (DMEM), with Earle's Balanced salt solution (ATCC) supplemented with 10% (v/v) Serum Supreme (Lonza, Biowhittaker) and 1% Penicillin/ Streptomycin (Gibco). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

All compounds were screened for cytotoxicity in the absence of light and after light activation. Light and dark toxicity screens were carried out on cell passage numbers between 7 and 15 in 96 well plates (Costar) in Optimem I Reduced Serum Medium with 1% Serum Supreme and 1% Penicillin/ Streptomycin (Gibco) in duplicate. The cells were seeded in the 96 well plates (50 μL) at a density of 1.5 x 10³ cells per well and were allowed to adhere for at least four hours before the addition of compound.

600 μM stock solutions of the ruthenium complexes were prepared in Optimem with 1% Serum Supreme on a separate 96 well plate. 220 μL were added to all other wells and 1:3 serial dilutions were performed by transferring 110 μL down each column of the 96 well plate to give the following compound concentrations: 600 μM, 200 μM, 67 μM, 22 μM, 7.4 μM, 2.4 μM, 0.8 μM, 0 μM. Once these dilutions were prepared, 50 μL of the metal solutions were transferred to the
96 well plate containing 50 μL of cells (1:2 dilution) to give the final ruthenium concentrations: 300 μM, 100 μM, 33.5 μM, 11 μM, 3.7 μM, 1.2 μM, 0.4 μM, and 0 μM.

During the addition of the ruthenium complexes to the cells, care was taken to protect the compounds from light. After compound addition, the plates were covered with aluminum foil to continue their protection from light and incubated at 37 °C with 5% CO₂ in a humidified atmosphere for 96 hours. Cell viability was subsequently measured with the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega). Viability was measured using a Tecan Spectrafluor Plus plate reader equipped with Magellan v7.0 software. The luminescent signal, due to the conversion of luciferin and ATP to oxyluciferin, AMP, and light, was measured after a five minute incubation of the cells with the cell-titer glo. During this time complete cellular lysis occurred allowing for maximal signal with minimal well to well variability.

Upon overnight incubation of the compounds in the absence of light, light activation studies were carried out using a 3M overhead projector (model 955) with an 82 V/ 410 W lamp (Model Osram FXL) fitted with a blue light cutoff filter (Edmund Optics, part # NT43-941) and a mirror angled at 45 degrees to reflect the light downward onto the 96 well plates. The 96 well plates were exposed to blue light for three minutes and returned to the incubator for 72 hours. Cell viability was measured with the aforementioned Promega Cell Titer-Glo Luminescent Cell Viability Assay Kit. IC₅₀ values were calculated utilizing Prism software with the curves fit to a Variable Slope, Log(inhibitor) vs. Response equation.
Chapter 5.2: *In Vivo* A549-GSH Cell Viability Assay: Experimental

A549 cells were plated in a similar fashion described in 5.1 at 1500 cells/well. Identical dark assay conditions as described in Ch. 5.1 Experimental were employed, but with the addition of L-glutathione (GSH). Experiments were carried out in triplicate using Cisplatin and GL002 to determine the effect GSH has on cell viability. Cisplatin and GL002's concentrations were held constant, at a concentration of 20 μM, and GSH was subsequently added in dose response at concentrations of 16 μM, 8 μM, 4 μM, 2 μM, 1 μM, 0.5 μM, 0.25 μM, and 0.125 μM. GL002 was light-activated after a 12 hour incubation, and viability determined after a total incubation time of 96 hours. The effect of GSH with cisplatin was measured after a 96 hour incubation with the cells. Viability was measured with the ATP Luciferase assay and the aforementioned Tecan plate reader at 96 hours. IC₅₀ values were calculated utilizing Prism software with the curves fit to a Variable Slope, Log(inhibitor) vs. Response equation.
Chapter 1 References:


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Chapter 2 References:


**Chapter 3 References:**


**Chapter 4 and 5 References:**

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